MOLECULAR ANALYSIS OF EXOPOLYSACCHARIDE GENES OF *RHIZOBIUM* SP. STRAIN NGR234

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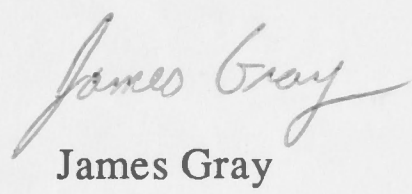
This thesis contains no work which has been used for the award of any other degree or diploma at any university. It contains no more than 100,000 words of text and contains no results generated by other persons except where due reference and acknowledgement has been made.



James Gray

For Suzanne and our children,

Jessica Lee and Ellie Rose.

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ABSTRACT

This thesis presents a molecular analysis of genes involved in the production of acidic exopolysaccharide (EPS) in the *Rhizobium* sp. strain NGR234. The Tn*5* insertion sites for 27 Exo$^-$ (deficient in EPS production) mutants, which belonged to six genetic complementation groups, were shown to be clustered over a 20 kb region of DNA. It was demonstrated previously that the mutant alleles belonging to two of the genetic groups were dominant to the wild-type allele in merodiploid strains where the mutant allele was carried on a plasmid. A detailed molecular examination of these mutants, presented here, revealed that their Tn*5* insertions were located within a single gene, termed *exoY*. It was also conclusively demonstrated that the dominant phenotype associated with these mutant *exoY*::Tn*5* alleles was in fact due to the expression of a neighbouring *exo* gene in the absence of *exoY*. The novel *exo* gene, termed *exoX*, conferred the dominant Exo$^-$ phenotype only when it was present in a copy number above that of *exoY* or when *exoY* had been mutated or deleted.

A significant level of DNA sequence homology was demonstrated between *exoX* and the *R. leguminosarum* biovar *phaseoli psi* gene, and similarly between *exoY* and the *R. l.* bv. *phaseoli pss2* gene. The *exoY* gene is part of a multi-cistron operon, of which part of the second gene, termed ORF1, was also sequenced. Cloned DNA fragments containing only *exoY* in the absence of down-stream sequences, were able to counter-act the EPS synthesis inhibition phenotype associated with *exoX*, verifying that the down-stream sequences were not required for the repression of the *exoX* phenotype. However, these sequences were necessary to counteract a deleterious nature associated with expression of *exoY* in their absence. A normal rate of EPS production by *R.* sp. NGR234 was dependent upon maintaining an equal copy-number of the *exoX* and *exoY* genes within the genome. A higher copy-number of *exoX* relative to that of *exoY* resulted in the inhibition of EPS synthesis.

The cluster of *exo* genes in *R.* sp. NGR234 (a nonasaccharide repeat unit) was found to have homology to the cluster of acidic EPS (succinoglycan) genes in *R. meliloti* strain SU47. The gene organization was similar and many of the genes were functionally interchangeable. However unlike *exoX*, which could efficiently inhibit EPS synthesis in several *Rhizobium* species, the activity of *exoY* was more dependent upon *exo* genes from its own cluster for efficient production of EPS. It is proposed that the products from *exoX, exoY*, ORF1 and perhaps other genes form a post-translational complex associated with the membrane. The *exoX* gene product would reversibly bind to the biosynthetic complex and inhibit EPS synthesis only while it was bound. The level of production of EPS in *Rhizobium* could be regulated by variations in the ratio of ExoX to ExoY. Attempts to find physical growth conditions that could regulate the expression of *exoX* or *exoY* were largely unsuccessful. However, the expression of *exoY* was shown to be affected by another *exo* gene that maps outside of the cluster and evidence is presented that *exoY* may also be regulated by a *nodD2*-like gene similar to that observed in *R. fredii*. Finally, the hybrid *Rhizobium* strains involving the *exo* DNA regions from *R.* sp. NGR234 and *R. meliloti* were also used to investigate the role of acidic EPS in determining host-recognition and in the development of a nitrogen-fixing nodule. The results indicated that the production of the homologous type of EPS was necessary for successful symbiosis.

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List of Abbreviations

Ap	ampicillin
BSA	bovine serum albumin
CFU	colony forming units
CIP	calf intestinal phosphatase
Cm	chloramphenicol
ddNTP	dideoxynucleoside triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EPS	exopolysaccharide
IPTG	isopropyl-β-D-thiogalactopyranoside
Km	kanamycin
LPS	lipopolysaccharide
MOPS	morpholinepropanesulfonic acid
OD	optical density
ONPG	*O*-nitrophenol-β-D-galactopyranoside
ORF	open reading frame
PBM	peribacteroid membrane
PBU	peribacteroid unit
PEG	polyethyleneglycol
PNK	polynucleotide kinase
RBS	ribosome binding site
Rif	rifampicin
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Sm	streptomycin
Sp	spectinomycin
ssDNA	single stranded DNA
Tc	tetracycline
TCA	trichloroacetic acid
Tris	Tris (hydroxymethyl) aminomethane
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

MOLECULAR ANALYSIS OF EXOPOLYSACCHARIDE GENES OF *RHIZOBIUM* SP. STRAIN NGR234

CHAPTER ONE
General Introduction

1.1 Introduction

Polysaccharides occur with a seemingly infinite array of chemical structures and collectively they are an important class of informational molecules involved in molecular and cellular interactions. Oligosaccharide molecules derived from degraded cell walls of fungi and plants have been identified as signals and regulatory molecules affecting expression of certain plant genes (reviewed by Albersheim and Darvill, 1985; Ryan, 1988). It is particularly relevant that some plant defensive genes are activated by small oligosaccharides that are not covalently attached to proteins. In addition, the signal molecule synthesized by *Rhizobium meliloti*, which causes root hair curling and cortical cell division on alfalfa roots, is a sulphated and acylated tetrasaccharide molecule (Lerouge *et al.*, 1990). Hence, an oligosaccharide-based communication system is fundamental to rhizobia-plant interactions.

1.2 *Rhizobium* Surface Polysaccharides

Wild-type *Rhizobium* cells often produce large quantities of extracellular polysaccharides and form very mucoid colonies on laboratory media. The major types of polysaccharide molecules found loosely attached to the bacterial outer membrane surface are the neutral β-1,2-glucans and the acidic exopolysaccharide (EPS). The lipopolysaccharides (LPS) which form part of the outer membrane and contain the O-somatic antigens, makes up the other major group of extracellular polysaccharides.

Neutral β-1,2-glucans are homopolymers consisting of β-(1→2)-linked glucose molecules with a degree of polymerization between 17 and 24 residues and possibly in a cyclic configuration (Dell *et al.*, 1983). Modified β-1,2-glucans have been reported for some *Rhizobium* species and two other polysaccharide producing soil bacteria of the

Agrobacterium genus. Modifications include: *sn*-glycerol-1-phosphates at the C6 position of some of the glucose residues of the β-1,2-glucans produced by *Rhizobium* sp. NGR234 (Batley *et al.*, 1987) and *Agrobacterium tumefaciens* (Miller *et al.*, 1987); succinic acid substitutions on the β-1,2-glucans produced by *Rhizobium leguminosarum* bv. *phaseoli* and *R. l.* bv. *trifolii*, and both methylmalonic acid and succinic acid on the β-1,2-glucans produced by *Agrobacterium radiobacter* (Hisamatsu *et al.*, 1987). Although neutral β-1,2-glucans are extracellular, a portion of the polysaccharide is found within the periplasmic space.

The acidic exopolysaccharides (EPS) are high molecular weight complex heteropolysaccharide polymers of a linked repeating unit that consists of 7,8 or 9 sugar residues. These oligosaccharide repeat units contain sugars linked by various α and β linkages in either a linear order or with branched side chains and quite often the sugars contain the non-carbohydrate substituents of succinate, pyruvate, acetate and hydroxybutanoate. There are more than a 100 different monosaccharides found in nature, yet relatively few are found in polysaccharide molecules. The more common sugars include hexoses; such as *D*-glucose, *D*-galactose, *D*-mannose, and the corresponding N-acetylaminosugars, as well as *L*-fucose and *L*-rhamnose (Sutherland, 1989). The acidic nature comes from the uronic acids, pyruvate ketals and succinyl non-carbohydrate substituents.

The EPS produced by *Rhizobium* sp. NGR234 has a nonasaccharide repeat unit that contains five glucoses, two galactoses and two glucuronic acids, all in various α and β linkages, and one pyruvate and one acetate group (Djordjevic *et al.*, 1986). The oligosaccharide repeat unit is structured such that it has a back-bone of six sugars and a branched side chain of three sugars, with the two non-carbohydrate substituents attached to the terminal galactose of the side chain (Fig. 1.1A). In comparison, the major acidic EPS (EPSa or EPS-I) produced by the most studied strain (SU47) of *R. meliloti* is a

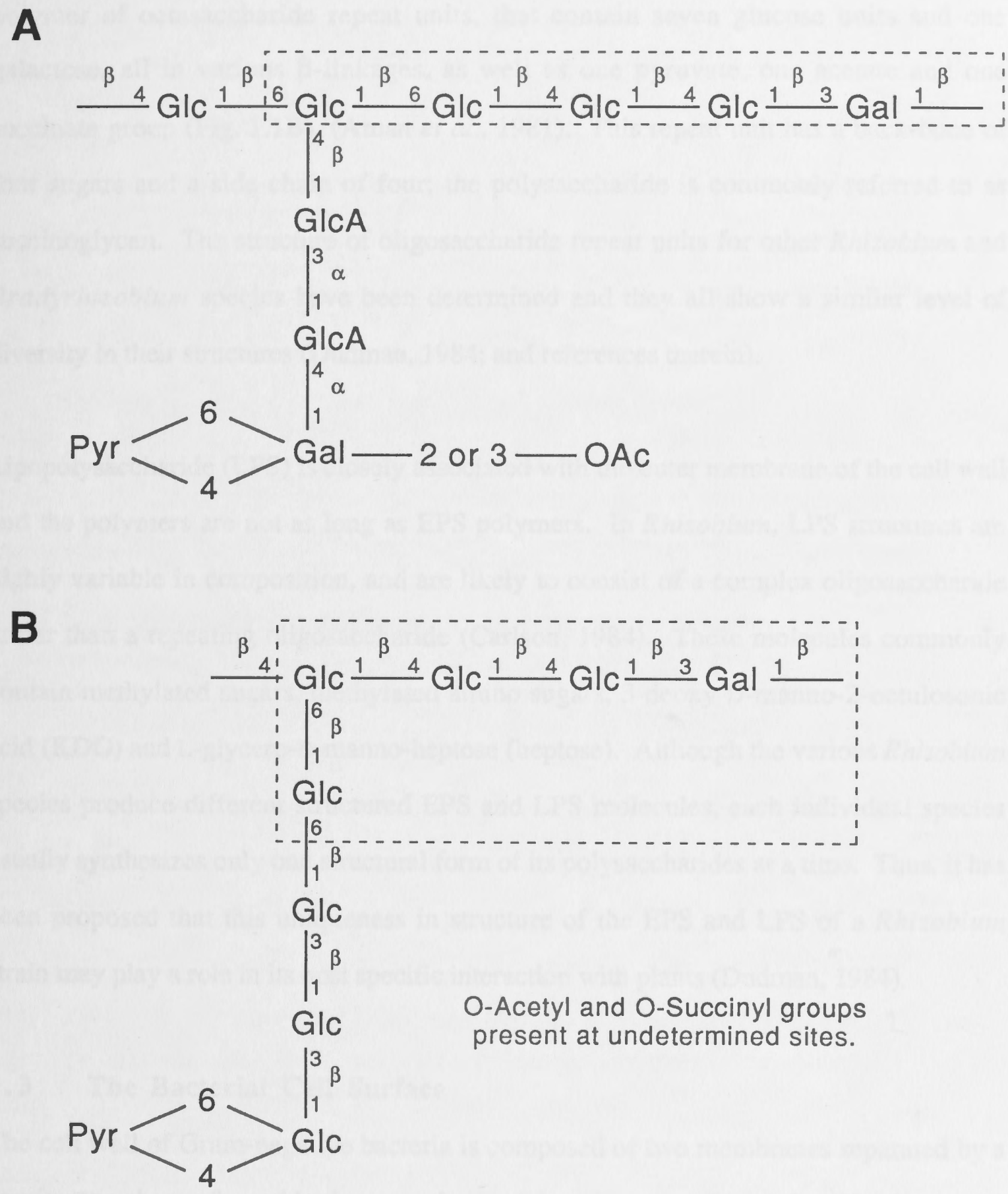


Fig. 1.1 Chemical structure of the oligosaccharide repeat units for the acidic EPS from **(A)** *R.* sp. NGR234 (Djordjevic, *et al.*, 1986) and **(B)** *R. meliloti* succinoglycan (Aman *et al.*, 1981). The five sugars and linkages enclosed within boxes are common to both oligosaccharide structures. Chemical abbreviations are: Glc, glucose; Gal, galactose; GlcA, glucuronic acid; Pyr, pyruvate; OAc, O-acetyl.

polymer of octasaccharide repeat units, that contain seven glucose units and one galactose, all in various β-linkages, as well as one pyruvate, one acetate and one succinate group (Fig. 1.1B), (Aman *et al.*, 1981). This repeat unit has a back-bone of four sugars and a side chain of four; the polysaccharide is commonly referred to as succinoglycan. The structure of oligosaccharide repeat units for other *Rhizobium* and *Bradyrhizobium* species have been determined and they all show a similar level of diversity in their structures (Dudman, 1984; and references therein).

Lipopolysaccharide (LPS) is closely associated with the outer membrane of the cell wall and the polymers are not as long as EPS polymers. In *Rhizobium*, LPS structures are highly variable in composition, and are likely to consist of a complex oligosaccharide rather than a repeating oligosaccharide (Carlson, 1984). These molecules commonly contain methylated sugars, methylated amino sugars, 3-deoxy-D-manno-2-octulosonic acid (KDO) and L-glycero-D-manno-heptose (heptose). Although the various *Rhizobium* species produce different structured EPS and LPS molecules, each individual species usually synthesizes only one structural form of its polysaccharides at a time. Thus, it has been proposed that this uniqueness in structure of the EPS and LPS of a *Rhizobium* strain may play a role in its host specific interaction with plants (Dudman, 1984).

1.3 The Bacterial Cell Surface

The cell wall of Gram-negative bacteria is composed of two membranes separated by a continuous sheet of peptidoglycan and a periplasmic space (Fig. 1.2). The inner cell membrane consists of two phospholipid layers, which are symmetrically opposed and form what is termed the lipid bilayer. Approximately 70% of the mass of the inner membrane is due to its protein constituents, which span the bilayer either partially or fully. These inner membrane proteins include specific enzymes and transport molecules that perform complex tasks including metabolic functions such as electron transport and oxidative phosphorylation, replication of DNA, specific transport of nutrients and ions,

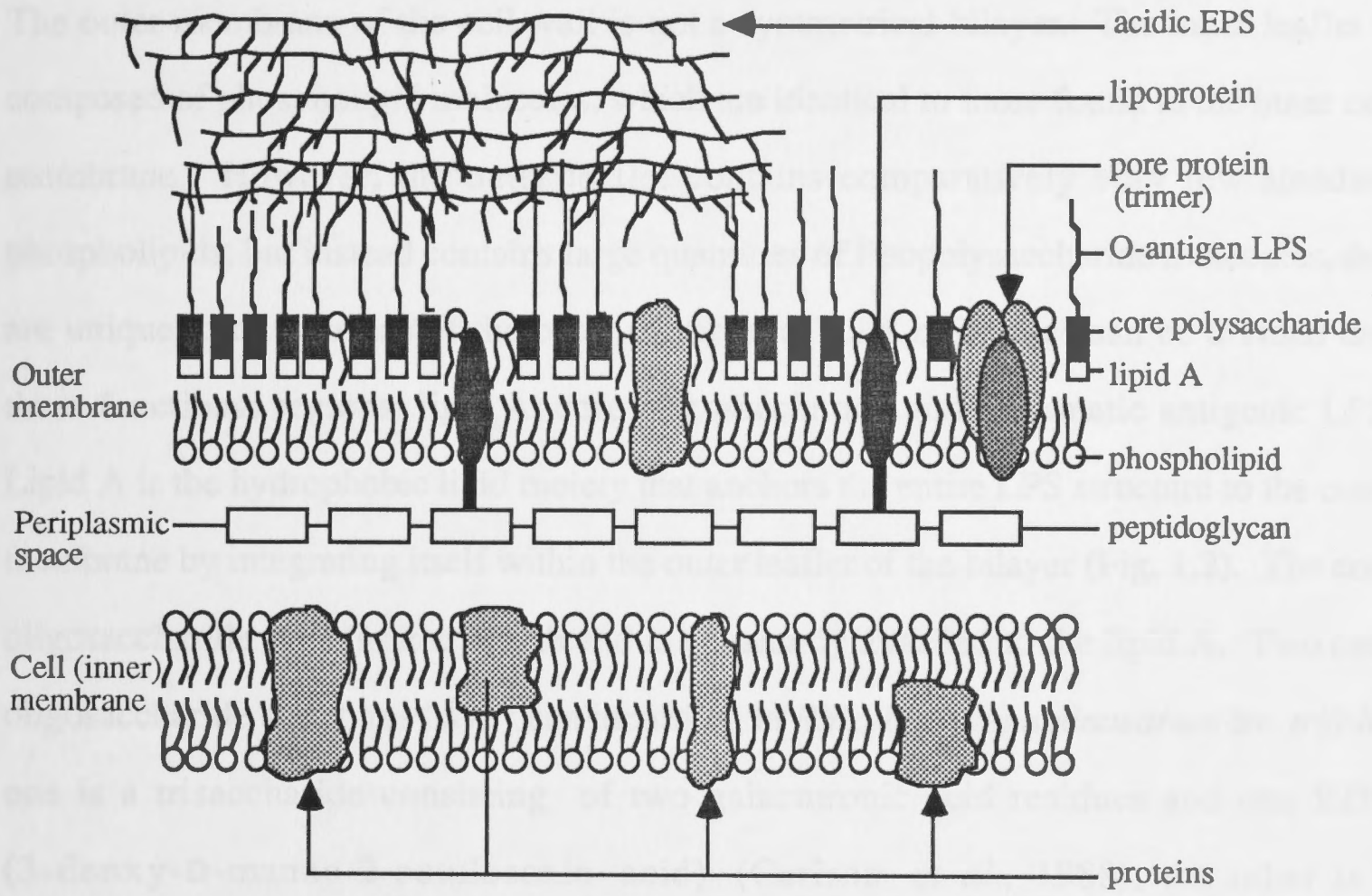


Fig. 1.2 Diagrammatic representation of the cell wall of a Gram-negative bacteria. The components of the double membrane complex are listed on the right. The outer surface of the outer membrane is composed mainly of lipopolysaccharide molecules and the acidic exopolysaccharide molecules are loosely attached to the protruding LPS chains by non-covalent associations. (The diagram has been modified from one that appears in Ingraham *et al.*, 1983, p 55, and therein referenced only as Davis, 1976).

secretion of proteins, transduction of sensory signals, synthesis of complex lipids, and synthesis of wall components including polysaccharides (Ingraham *et al.*, 1983).

The outer membrane of the cell wall is not a symmetrical bilayer. The inner leaflet is composed of phospholipid molecules, which are identical to those found in the inner cell membrane. However, the outer leaflet contains comparatively very few standard phospholipids, but instead contains large quantities of lipopolysaccharide molecules, that are unique to this surface of the outer membrane. LPS molecules can be divided into three functional regions; lipid A, core oligosaccharide and O-somatic antigenic LPS. Lipid A is the hydrophobic lipid moiety that anchors the entire LPS structure to the outer membrane by integrating itself within the outer leaflet of the bilayer (Fig. 1.2). The core oligosaccharide is of limited length and is covalently attached to the lipid A. Two core oligosaccharide structures have been identified in *Rhizobium leguminosarum* bv. *trifolii*; one is a trisaccharide consisting of two galacturonic acid residues and one KDO (3-deoxy-D-manno-2-octulosonic acid) (Carlson *et al.*, 1988), the other is a tetrasaccharide consisting of galactose, galacturonic acid, mannose and KDO (Holingsworth *et al.*, 1989). Attached to the core polysaccharide is the polymorphic region of the LPS (O-antigen), which projects outwards from the cell surface. The acidic exopolysaccharide (EPS) forms a final encapsulating layer around the *Rhizobium* cell, by loosely binding via non-covalent associations to the protruding LPS chains.

The outer membrane also contains a number of proteins, but comparatively fewer than is found on the inner membrane. The main protein constituents are lipoprotein, pore protein and outer membrane protein. The lipoprotein is the most abundant; it fully spans the outer membrane and is covalently attached to the peptidoglycan layer via a disulfide bond. The peptidoglycan layer gives the cell wall its rigid qualities that enable it to withstand turgor pressure, while simultaneously it is fully permeable.

1.4 Carbohydrate Signals in Plant-Microbe Interactions

Oligosaccharides are an important class of signal molecules, which in many instances, have been shown to regulate gene expression in plants (reviewed by Hahn *et al.*, 1989; Ryan, 1988). Oligosaccharides derived from fungal, bacterial and plant origins have been identified, that can act as chemical signals to activate a wide array of plant genes that are involved in numerous and varied responses, such as plant defence and developmental differentiation. Most importantly, these oligosaccharides act on their own and not in association with proteins (Rademacher *et al.*, 1988), unlike the glycoprotein recognition systems operating in animals and yeast.

Oligosaccharide molecules that have originated from degraded fungal cell wall polysaccharides, have been shown to elicit phytoalexin accumulation in plant cells (Darvill and Albersheim, 1984). Phytoalexin production is a standard defense response by plants when challenged by a pathogen and results from *de novo* synthesis of the necessary biosynthetic enzymes. Biologically active oligosaccharides (elicitors) have precise structures and stimulate phytoalexin accumulation when present at concentrations of 10^{-8} to 10^{-9} Molar, while closely related structural isomers are inactive at even higher concentrations. An example of this is a branched hepta-β-glucoside isolated from cell walls of *Phytophthora megasperma* f.sp. *glycinea* (Sharp *et al.*, 1984). While one hepta-β-glucoside structure was shown to be active, about 300 other hepta-β-glucosides (including structurally related isomers) that varied with respect to their branch locations and linkages, were inactive. The precise structural requirements for elicitor activity suggests that the corresponding host-plant cells (soybean cotyledons) have highly discriminating receptors specific for this elicitor (Sharp *et al.*, 1984).

Oligosaccharide signal molecules can also originate from the cell walls of the plant cells they affect. Primary cell walls of plants are a complex mixture of glycoproteins and polysaccharides, of which approximately 70% are complex carbohydrates (Ryan, 1988).

Oligosaccharide fragments generated by degradation of plant cell walls by endopolygalacturonases, pectic lyases or by mild acid hydrolysis, may also act as inducers of the same defensive responses in plants that are elicited by β-glucans from fungal cell walls (Ryan, 1988). These plant cell wall fragments with signal activity were a variety of small α-1,4-galacturonic acid oligomers derived from the backbone of the pectin components of the plant cell walls. In an *in vivo* situation, these fragments are generated in response to plant pathogenesis, as most phytopathogenic fungi and bacteria produce a variety of cellulose and pectin-degrading enzymes capable of depolymerizing the polysaccharides of plant cell walls (Cooper, 1984; Hahn *et al.*, 1989).

In addition to the elicitation of plant defense systems, oligosaccharides have also been shown to be important intercellular signals that regulate growth and differentiation of plant tissues. For instance, a particular nonasaccharide fragment derived from enzymatic digestion of xyloglucan (found in plant cell walls) , was shown to inhibit the 2,4-dichlorophenoxyacetic-acid-induced elongation of etiolated pea stem segments, when present at 10^{-9} M (McDougall and Fry, 1988). In another example, pectic cell wall fragments were shown to inhibit the formation of roots on tobacco explants grown on a root inducing medium (Eberhard *et al.*, 1989). In both of these examples, the pectic fragments regulate plant morphogenesis with effects that are similar to those induced by auxin and cytokinin, but by a unique, yet unknown mechanism (Eberhard *et al.*, 1989).

The report of an oligosaccharide-based communication system, which is applicable to this thesis, is that by Lerouge *et al.* (1990). Lerouge *et al.* (1990) demonstrated that the active determinant of host-range between *Rhizobium meliloti* and its symbiotic host (see 1.5), alfalfa, is an oligosaccharide synthesized by the bacteria. The molecule, termed NodRm-1, is a sulphated β-1,4-tetrasaccharide of D-glucosamine in which three amino groups are *N*-acetylated and one was acylated with a C_{16} unsaturated fatty acid. This oligosaccharide was able to induce cortical cell division and distortion of root hair cells of

alfalfa plants, which are amongst the earliest visual signs of nodule organogenesis (see 1.6). Clearly, more signals are necessary for complete nodule development and many of these probably also originate from the *Rhizobium*. Certain alfalfa varieties are able to develop non-nitrogen-fixing nodules in the absence of *Rhizobium* (Truchet *et al.*, 1989), indicating that the plant possesses all of the genetic hardware for nodule development, but requires *R. meliloti* as a source of stimulatory molecules. Synthesis of the bioactive oligosaccharide signal molecule required *nodC*, which was not surprising since *Rhizobium nodC* mutants are unable to induce root hair curling. However, the molecule could be synthesized by *exoB* mutants of *R. meliloti*, which are unable to synthesize either of the two acidic exopolysaccharides (Glazebrook and Walker, 1989; Zhan *et al.*, 1989) and it produces lipopolysaccharide that has a structure different to that synthesized by the wild-type *R. meliloti* strain (Leigh and Lee, 1988). Thus the biosynthetic pathway for NodRm-1 oligosaccharide synthesis is likely to diverge quite early from the anabolic pathway that leads to exopolysaccharide and lipopolysaccharide production.

1.5 The Legume-*Rhizobium* Symbiosis

The Leguminosae family is the third largest plant family in the Angiosperms, containing an estimated 16,000 to 19,000 species in about 750 genera, ranging from annual herbs to large trees (Allen and Allen, 1981). Habitats for members of the Leguminosae family spread from the arctic to equatorial tropical and desert regions. Legumes are essentially unique in their ability to form symbiotic associations with soil bacteria of the genera *Rhizobium* and *Bradyrhizobium*, which are rhizobia that are aerobic, Gram negative bacteria that are readily isolated from the nodule structures found on the root systems of legumes.

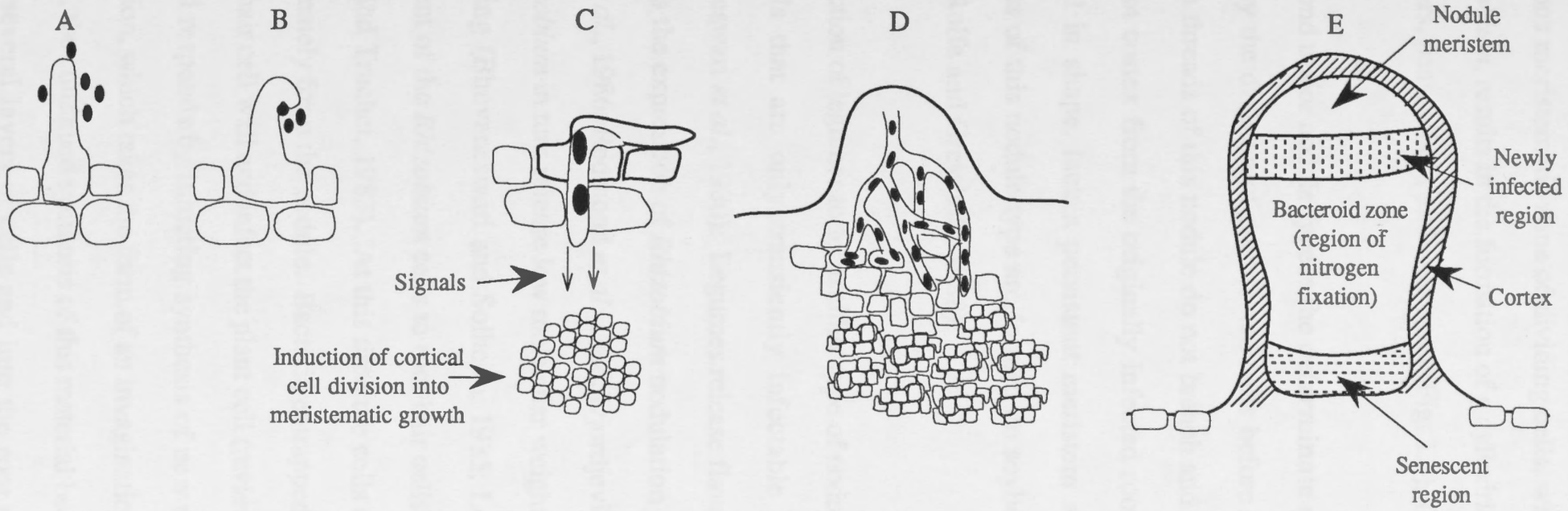
The symbiotic association between rhizobia and legumes gives both partners a decided survival advantage. The bacteria live in a sheltered environment (the nodule) where they receive a supply of fixed carbon and other nutrients; and in exchange, the rhizobia export

fixed nitrogen for use by the plant. Biological nitrogen fixation is the conversion of atmospheric nitrogen (N_2) to ammonia, by a process involving various nitrogenase enzymes that are encoded within the bacterial genome (eg. Kurz and LaRue, 1975; McComb *et al.*, 1975; Pagan *et al.*, 1975). Nitrogen is an essential constituent of the proteins necessary for cell metabolism, thus all organisms require it in a utilizable form. Unless plants are able to procure their fixed nitrogen from an association with nitrogen-fixing bacteria, they must rely on combined forms of N present in the soil.

With few exceptions, particular *Rhizobium* species are able to form nodules on (nodulate) roots from a narrow range of plant hosts; referred to as "cross-inoculation groups". Examples of commonly researched cross-inoculation groups are: *Rhizobium meliloti* and alfalfa (*Medicago sativa*), *R. fredii* and soybean (*Glycine max*), *R. leguminosarum* bv. *viciae* and peas (*Pisum sativum*), *R. l.* bv. *phaseoli* and beans (*Phaseolus* spp.), *R. l.* bv. *trifolii* and clovers (*Trifolium* spp.), *Bradyrhizobium japonicum* and soybean (Allen and Allen, 1981). An exception to the narrow host range exhibited by other *Rhizobium* species is *R.* sp. NGR234, which is capable of nodulating 23 known species including the non-legume woody tree *Parasponia andersonii* (Trinick, 1973; Trinick, M. J. and J. Galbraith, 1980). *Parasponia* is the only non-legume genus that has been found to undergo such a symbiosis with rhizobia.

1.6 Nodule Ontogeny and *Rhizobium* Infection

Two basic types of nodule developmental patterns are recognized (Sprent, 1979; Bergersen, 1982). Infection of new cells within **indeterminate** nodules occurs by means of infection threads, which first arise from the infected root hair cell, then pass through the outer cortical cells and ramify in the root cortex (Fig. 1.3). In the inner cortex, cells are induced to divide and form a focus of dividing plant cells ahead of the advancing infection thread. Those proliferating plant cells that are invaded by infection threads, cease to divide and begin to enlarge and swell. An apical meristem is formed by



(A) Bacterial adhesion to emerging root hair.	(B) Pronounced deformation and curling of the root hair caused by extracellular *Rhizobium* signals.	(C) Bacterial invasion via infection thread. Signals may be of *Rhizobium* origin or secondary signals of plant origin, resulting from a transduction of the primary *Rhizobium* signal (Long and Cooper, 1988).	(D) Infection thread ramifies and invades cortical cells, bacteria are released into cortical cells that have ceased dividing, while meristematic growth continues ahead of the advancing infection thread.	(E) Nitrogen fixing nodule.

Fig. 1.3 Schematic representation of developmental steps in the formation of an indeterminate nodule.

the adjacent uninvaded cells which continue to divide. The maintenance of this continuous meristematic zone of dividing cells, where only some cells become infected by *Rhizobium*, results in the formation of a cylindrical indeterminate nodule; as observed on clovers, peas, alfalfa and *Leucaena* (Fig. 1.3), (Rolfe and Gresshoff, 1988).

The second major nodule type is the **determinate** nodule, where the bacteria are mainly spread by the division of preinfected cells before they cease to divide and swell. The infection threads of this nodule do not branch and ramify extensively after entering the outer root cortex from the originally infected root hair cell. These nodules are more spherical in shape, lack a persistent meristem and have a closed vascular system. Examples of this nodule type are formed on soybeans, *Phaseolus* beans, cowpeas and siratro (Rolfe and Gresshoff, 1988).

The infection of legumes to form either type of nodule is usually initiated at emerging root hair cells that are only transiently infectable for periods as short as six hours (Bhuvaneswari *et al.*, 1980). Legumes release flavanoid compounds and these induce or derepress the expression of *Rhizobium* nodulation genes (see 1.7), (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.* 1986; Djordjevic *et al.*, 1987b; Horvath *et al.*, 1987). The *Rhizobium* in turn release low molecular weight factors that cause root hair distortion and curling (Bhuvaneswari and Solheim, 1985; Lerouge *et al.*, 1990). In some cases, attachment of the *Rhizobium* cells to root hair cells is mediated by plant secreted lectins (Dazzo and Truchet, 1983). At this time the cells of the root cortex begin dividing and will ultimately form the nodule. Bacteria entrapped within curled root hair cells degrade the root hair cell wall and infect the plant cell (reviewed by Djordjevic *et al.*, 1987a). The plant cell responds by initiating synthesis of new cell wall material beneath the site of degradation, which takes the form of an invagination. As *Rhizobium* cells continue their infection, the continued synthesis of this material becomes an infection thread, that passes through several layers of cells and into the root cortex (in the case of indeterminate

nodulation). Within the root cortex, the bacteria are released into the cytoplasm of cortical cells and are surrounded by a membrane (the periplasmic membrane) derived from the plant plasmalemma (Paau *et al.* 1978). The bacteria continue one or two cell divisions and then they differentiate into the nitrogen fixing bacteroids and exchange ammonia for photosynthate and other nutrients from the host.

1.7 Bacterial Genetics Contributing to Nodule Development

A wide array of *Rhizobium* genes are required for nodule morphogenesis and many of these have been categorized as genes involved in: nitrogen fixation (*nif* and *fix* genes), nodulation (*nod* genes), host range or host specific nodulation (*hsn* genes), exopolysaccharide synthesis (*exo* genes) (recent reviews: Djordjevic *et al.*, 1987a; Rolfe and Gresshoff, 1988; Downie and Johnston, 1988; Long, 1989; Kondorosi *et al.*, 1989a). Many of these genes occur in clusters on large indigenous plasmids, which are usually several hundred kilobases (kb) in size. In fast-growing *Rhizobium* species, it has been shown that at least some *nod, hsn, fix*, and *nif* genes map to one of these large indigenous megaplasmids and this plasmid has been referred to as the symbiotic plasmid or pSym (Beringer *et al.*, 1980; Brewin *et al.*, 1980; Banfalvi *et al.*, 1981; Rosenberg *et al.*, 1981; Djordjevic *et al.*, 1983).

Three nodulation genes (*nodA, nodB* and *nodC*) that are common to many *Rhizobium* species, are transcribed from a single operon, referred to as *nodABC*. Mutations in any of these genes block nodule development at the earliest step, root hair curling and cortical cell division (Rossen *et al.*, 1984; Kondorosi *et al.*, 1984; Djordjevic *et al.*, 1985b; Bender *et al.*, 1987;Dudley *et al.*, 1987). These "common nodulation" genes show a high degree of sequence similarity and are functionally interchangeable among all *Rhizobium* species, without affecting their host range (Kondorosi *et al.*, 1984; Djordjevic *et al.*, 1985a; Fisher *et al.*, 1985; Marvel *et al.*, 1985). The *nodABC* operon is not expressed in cultured cells, but is induced by another gene, *nodD*, in the presence of

plant exudates or extracts (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985). Inducing compounds have been identified for several systems as; 7,4'-dihydroxyflavone in clover (*T. repens*) (Redmond *et al.*, 1986), 3',4',5,7-tetrahydroxyflavone in alfalfa (Peters *et al.*, 1986), apigenin-7-O-glycoside in peas (Firmin *et al.*, 1986), and in most cases they are hydroxylated flavones or flavanones. Expression of *nodD* is constitutive or in the case of *R. l.* bv. *viciae*, is autogenously regulated by its own transcription (Rossen *et al.*, 1985). The *nodABC* operon is activated by the *nodD* gene product when in the presence of the plant secreted inducer compounds mentioned above and in the case of *R. meliloti*, the divergent promoters of *nodD1* and *nodABC* are also transcriptionally repressed by another repressor protein (Kondorosi *et al.*, 1989b). In *R. meliloti* there are three functional copies of the *nodD* gene on the symbiotic plasmid (Göttfert *et al.*, 1986; Honma and Ausubel, 1987), which demonstrate diverged flavonoid-recognizing abilities and they each affect the activation of *nod* genes in a host-dependent manner (Horvath *et al.*, 1987; Györgypal *et al.*, 1988). Altered *nodD* genes created by point mutations or by recombination to form chimeras, can in some instances result in altered regulatory traits and recognition of inducer molecules and can even extend the host-range of its *Rhizobium* (Burn *et al.*, 1987; McIver *et al.*, 1989; Spaink *et al.*, 1989a and 1989b).

In *R. leguminosarum* biovars there are two additional *nod* genes, *nodI* and *nodJ*, located down-stream of *nodC* in the same operon (Evans and Downie, 1986), and mutants at these loci are slightly affected with regard to the onset and extent of nodulation of some symbioses (Djordjevic *et al.*, 1985a) although not in others (Knight *et al.*, 1986). All *Rhizobium* species have other *nod* genes grouped into several operons, such as *nodFEL, nodG, nodH*, and *nodMN* (reviewed by Downie and Johnston, 1988; Rolfe and Gresshoff, 1988; Long, 1989). Mutations in any of these genes do not totally inhibit nodulation, but do result in abnormal root hair reactions on their usual hosts and occasionally elicit root hair reactions on heterologous hosts (Djordjevic *et al.*, 1985a;

Debellé *et al.*, 1986). Some of these genes are not conserved between *Rhizobium* species, as there is an absence of interspecies hybridization and the alleles do not substitute for one another when different host plants are involved (Kondorosi *et al.*, 1984; Djordjevic *et al.*, 1985a). Due to these host-range qualities, they are often referred to as host specific nodulation (*hsn*) genes. Many of these other operons, such as *nodFE* (*hsnAB*), *nodH* (*hsnD*) and *nodMN*, are also inducible by the same flavone-mediated, host specific, *nodD* activation system that regulates *nodABC* (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985; Shearman *et al.*, 1986; Gerhold *et al.*, 1989). In addition to the *nodABC* operon, the *nodH* (*hsnD*) and *nodQ* genes are also required for the synthesis of extracellular signals by *R. meliloti* that induce root hair deformation and cortical cell division on alfalfa (Faucher *et al.*, 1989; Banfalvi and Kondorosi, 1989; Lerouge *et al.*, 1990). The promoters of all operons that are activated by *nodD*, have an approximately 50 base pair (bp) DNA sequence (*nod*-box) that is significantly conserved between promoters and between *Rhizobium* species (Rostas *et al.*, 1986; Schofield and Watson, 1986; Shearman *et al.*, 1986; Fisher *et al.*, 1987). The *nod*-box is the target site for the *nodD* gene product (Hong *et al.*, 1987; Fisher *et al.*, 1988; Kondorosi *et al.*, 1989b) and it is clear that that the entire system functions to coordinately regulate transcription of the relevant *nod* and *hsn* genes.

1.8 Involvement of Polysaccharides in Nodule Development and Host-Range

Many studies have shown that acidic EPS is essential for establishing a nitrogen-fixing (Nod^+Fix^+) symbiosis on legumes that develop an indeterminate type of nodule, such as *R. meliloti* and alfalfa (e.g. Leigh *et al.*, 1985), *R. l.* bv. *viciae* and peas (e.g. Borthakur *et al.*, 1986), *R. l.* bv. *trifolii* and clover (Chakravorty *et al.*, 1982), and *R.* sp. NGR234 and *Leucaena leucocephala* (Chen *et al.*, 1985). In marked contrast, are Exo^- (defective in EPS synthesis) mutants of *R. fredii* and *R. l.* bv. *phaseoli*, which form normal nitrogen-fixing nodules, of the determinate type, on soybeans and

Phaseolus beans respectively (Kim *et al.*, 1989; Borthakur *et al.*, 1986; Diebold and Noel, 1989). Diebold and Noel (1989) demonstrated that Exo$^-$ mutants of *R. l.* bv. *phaseoli* were fully effective on beans, and that the identical mutations in *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* were also Exo$^-$, but formed defective nodules on peas and clovers respectively. These observations show that EPS is required for nodulation of the indeterminate type, but it is not necessary for the determinate type of ontogeny. Lipopolysaccharides (LPS) play a role in the infection of rhizobia and establishment of determinant type nodules. Mutants of *R. l.* bv. *phaseoli*, defective in their production of LPS, were incapable of normal nodulation on beans (Cava *et al.*, 1989; Diebold and Noel, 1989). Microscopic analysis showed that meristematic activity and infection thread formation was aborted soon after their initiation (Cava *et al.*, 1989).

On the whole, those Exo$^-$ mutants of *Rhizobium* that are unable to form nitrogen-fixing nodules, can be intercellularly complemented by Exo$^+$Nod$^-$ mutants of the same wild-type strain, when coinoculated on the host. For instance, Exo$^-$ mutants of *R.* sp. NGR234 that were able to curl root hairs, but were incapable of forming nodules were coinoculated with a Nod$^-$ mutant of *R.* sp. NGR234 that had been cured of its symbiotic plasmid (consequently lacking the essential *nod* genes); together they could form nitrogen fixing nodules on *Leucaena* (Chen and Rolfe, 1987). Similar coinoculation experiments between Nod$^+$Exo$^-$ and Nod$^-$Exo$^+$ mutants have resulted in functional nodules on clover by *R. trifolii* (Rolfe *et al.*, 1980; Chakravorty *et al.*, 1982), on peas by *R. l.* bv. *viciae* (Borthakur *et al.*, 1988), and on alfalfa by *R. meliloti* (Klein *et al.*, 1988, Müller *et al.*, 1988). In the case of pea nodules, the majority of bacteroids originated from the Exo$^+$ coinoculant (Borthakur *et al.*, 1988). Klein *et al.* (1988) conducted a detailed examination of the occupants from alfalfa nodules resulting from similar coinoculations of defective, but complementary strains of *R. meliloti*. Their results were interesting in that Nod$^+$Fix$^+$Exo$^-$ mutants could be assisted into forming a Fix$^+$ nodule by a Nod$^-$Fix$^-$Exo$^+$ helper strain, but not by a Nod$^+$Fix$^-$Exo$^+$ helper strain or even the wild-

type strain. For an effective combination, one of the coinoculants must provide the Nod$^+$ phenotype and the other the Exo$^+$ phenotype. If both of these functions can be provided by a single strain then the Exo$^-$ participant is excluded from the infection, perhaps by an inability to compete due to the necessity to acquire one of its prerequisites from intercellular complementation (Klein *et al.*, 1988). Further to these types of experiments, the ability of Exo$^-$ mutants of *R. l.* bv. *trifolii* and *R.* sp. NGR234 to induce nitrogen fixing nodules on clover and *Leucaena* respectively, can be restored by the accompaniment of purified EPS or oligosaccharide from their respective wild-type strains (Djordjevic *et al.*, 1987c). Thus, the Exo$^-$ mutants were able to provide early nodulation signals, but clearly for indeterminate nodulation, EPS is required for the continual progression of the infection thread.

Several lines of evidence suggest that exopolysaccharides also play a role in host-range determination. *R. meliloti* is known to synthesize two structurally different forms of acidic exopolysaccharide (Glazebrook and Walker, 1989; Zhan *et al.*, 1989), of which EPSb (EPS-II) is able to substitute for EPSa (EPS-I or succinoglycan) in the development of nitrogen-fixing nodules on *Medicago sativa* (alfalfa), but EPSb will not substitute for EPSa on four other plants that are normally hosts for this strain of *R. meliloti* (Glazebrook and Walker, 1989). Furthermore, the oligosaccharide signal that causes root hair curling on alfalfa plants (Lerouge *et al.*, 1990) (outlined in section 1.4), was shown to be specific for this host only, as it was unable to induce any response on heterologous host plants. Similarly, restoration of a nitrogen-fixing capacity to Exo$^-$ mutants of *R. l.* bv. *trifolii* and *R.* sp. NGR234 by the addition of purified EPS or oligosaccharide, was only successful when the polysaccharide was isolated from the corresponding parental wild-type strain and not when it was heterologous polysaccharide (Djordjevic *et al.*, 1987). The data suggests that unique oligosaccharide-receptor recognition systems exist between different sets of symbiotic partners.

In symbiotic systems where mutants of *R. meliloti* form an acidic polysaccharide which is slightly different to the wild-type succinoglycan, no nitrogen-fixing nodules resulted (Leigh *et al.*, 1987; Müller *et al.*, 1988). For instance, *exoH* mutants of *R. meliloti* (Leigh *et al.*, 1987) produce only large molecular weight (MW) polymers of acidic EPS, that was shown by proton NMR spectroscopy to lack the *O*-succinyl substituent, but was otherwise completely normal in sugar composition and linkages. These *exoH* mutants were defective in nodule invasion and the growth of infection threads. Similarly, *R. meliloti* mutant, strain 101.45 (Müller *et al.*, 1988), was shown by ^{13}C NMR spectroscopy to synthesize an EPS that lacked the terminal pyruvate substitution on its repeat unit side chain, but was otherwise normal in structure; although, the mutant produced excessive amounts of the EPS. Not only was mutant 101.45 defective in symbiosis, but it was unable to intercellularly complement normal EPS producing Nod$^-$ *R. meliloti* mutants (Müller *et al.*, 1988). Thus, it is evident that the host does not recognize the altered EPS and moreover, may even be able to sense a lower level of the pyruvate additions and subsequently block infection.

There is one example reported recently, of a *R. meliloti* strain where the functions of acidic EPS appear to be substituted for, by modified LPS molecules (Williams *et al.*, 1990). Strain AK631 is a spontaneous Exo$^-$ derivative of the wild-type isolate *R. meliloti* strain Rm41, which is different in several respects to the wild-type strain SU47, including the expression of an *lpsZ* gene in strain Rm41, which is apparently absent in strain SU47. The mutation in strain AK631 appears to be within the *exoB* gene (Leigh *et al.*, 1985), but instead of having an Inf$^-$Fix$^-$ phenotype on alfalfa which is normally associated with *exoB* mutants of strain SU47, strain AK631 is instead Fix$^+$ on alfalfa. It was proposed by Williams *et al.* (1990) that a strain Rm41 gene, *lpsZ*, which is not expressed in strain SU47, was responsible for the production of altered LPS molecules that substitute for the absence of acidic EPS during the infection of alfalfa by

strain AK631 *exoB* mutants. However, the structure of these proposed LPS signal molecules has not been determined as yet.

Neutral β-1,2-glucans, oligosaccharide repeat units and LPS have all been shown to enhance infection and nodulation when used to pretreat the roots of compatible hosts prior to inoculation with live cells (reviewed by Halverson and Stacey, 1986). However, sometimes it was necessary to isolate the active polysaccharide molecules from *Rhizobium* cultures that had been grown in the presence of root exudates from the host plant, suggesting that signals from the host may lead to modifications in the surface polysaccharides (Bhagwat and Thomas, 1984).

Neutral β-1,2-glucan may have a universal role in the interactions of both *Rhizobium* and *Agrobacterium* with their host plants. Mutations that lead to an absence of β-1,2-glucan synthesis, but do not affect EPS, have been characterized in *R. meliloti* and a derivative of *A. tumefaciens* (containing the pSym. from *R. l.* bv. *phaseoli*), which consequently nodulates *Phaseolus* beans; in both cases the mutants were incapable of nodulating their respective hosts (Hooykaas and Schilperoort, 1986; Cangelosi *et al.*, 1987). In addition, mutations at homologous loci in *A. tumefaciens* (*chvA* and *chvB*) resulted in avirulent, attachment-defective mutants of *A. tumefaciens* (Puvanesarajah *et al.*, 1985). This polysaccharide is a homopolymer, about 20 residues in length and consisting solely of β-(1→2)-linked glucose sugars (see 1.2). Although it can be modified in various ways, there is less scope for variation with this polysaccharide as compared to EPS or LPS. The function of β-1,2-glucans is likely to be passive and may be involved in the bacterial cell's adaptation to osmotic variations in its surroundings (Dylan *et al.*, 1990).

1.9 Lectin-Polysaccharide Interactions

An accumulation of evidence exists, in support of the hypothesis that host plant lectins have stringent specificity for the surface polysaccharides of *Rhizobium* cells within their

cross-inoculation groups (reviewed by Halverson and Stacey, 1986). Trifoliin A is a clover lectin isolated from root hairs, that specifically binds to the LPS and EPS of *R. l.* bv. *trifolii* (Dazzo and Truchet, 1983). Trifoliin A also binds to certain carbohydrate structures on clover roots and may function as a cross-bridge to intimately link *R. l.* bv. *trifolii* cells to the clover root hair cells. Thus, consequently leading to the induction of specific physiological responses and gene expression that are required for successful infection (Dazzo and Truchet, 1983). During the normal infection process the root hair cell lectins may also serve to hold the *Rhizobium* cell fixed at the tip of the root hair cell so that an efficient exchange of signals between the two organisms can occur.

Root lectins that specifically recognize their microsymbiont *Rhizobium* cells have also been demonstrated for soybean, sweet clover (*Melilotus alba*) and pea (reviewed by Halverson and Stacey, 1986). Recently, the gene encoding the pea root hair lectin was cloned and transferred to white clover using *Agrobacterium rhizogenes* as the vector (Diaz *et al.*, 1989). Nodule formation on these transgenic clover roots by *R. l.* bv. *trifolii* was not affected, but they were now susceptible to infection by *R. l.* bv. *viciae*, which induced a low level of delayed nodulation. The majority of the nodules formed by *R. l.* bv. *viciae* were abnormal, but electron microscopic examination revealed bacteria within intracellular spaces of the cortex, aborted infection threads and degenerated nodule meristems. Those nodules that did develop normally and fix atmospheric nitrogen, had normal nodule ultrastructures as determined by electron microscopy. Diaz *et al.* (1989) concluded that the host range in *Rhizobium*-legume symbiosis is at least partially determined by symbiont-root-lectin interactions.

In *R. l.* bv. *viciae,* the structure of its EPS, its host-range and its lectin binding properties are all altered by the addition of donor *R. l.* bv. *trifolii* host range genes (Philip-Holingsworth *et al.*, 1989b). The structure of the oligosaccharide repeat units for *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* strains differ only in the degree of substitution of

3-hydroxybutyrate and acetate (Philip-Holingsworth *et al.*, 1989a). When a plasmid carrying cloned *R. l.* bv. *trifolii* host specific nodulation (*hsn*) genes *nodFERLMN*, was transferred into *R. l.* bv. *viciae*, the resulting hybrid synthesized EPS identical to that of *R. l.* bv. *trifolii*. Furthermore, the hybrid was now able to nodulate clover and bind the clover lectin trifoliin A (Philip-Holingsworth *et al.*,1989b).

1.10 Biosynthesis of Bacterial Exopolysaccharides

The synthesis of acidic exopolysaccharide requires a supply of nucleoside diphosphate monosaccharides, cytoplasmic and membrane-associated biosynthetic enzymes, and takes place at the cytoplasmic side of the cell membrane (reviewed by Sutherland, 1982, 1985 and 1989). The octasaccharide repeat unit of EPS from *R. meliloti*, involves the incorporation of glucose, galactose, pyruvic acid and *O*-acetyl groups; and EPS biosynthesis in general, utilizes the appropriate sugar nucleoside diphosphate precursors, acetyl CoA, phosphoenolpyruvate and an isoprenoid phospholipid carrier (Tolmasky et al., 1980 and 1982; Sutherland, 1985). Assembly of the biological oligosaccharide repeat unit, follows an ordered process (Fig. 1.4); with the transfer of a sugar residue from a UDP-sugar to the lipid carrier in the membrane, followed by the addition of another sugar from another UDP-sugar residue via a separate transferase enzyme and so on, until the repeat unit is completed. Synthesis of oligosaccharide repeat units take place at the cell membrane, as lipid bound intermediates of EPS biosynthesis can be found in many bacteria (Sutherland, 1985) and in *R. meliloti* (Tolmasky *et al.*, 1982). In experiments where radioactive labelling was used to monitor the assembly of the succinoglycan (*R. meliloti* EPS) repeat unit (Tolmasky *et al.*, 1980 and 1982), it was demonstrated that the galactose became lipid bound first, followed by the β-(1→3)-linked glucose and the other six glucose residues were added on in subsequent steps (Fig. 1.4). The succinylated, acetylated and pyruvylated substitutions were occasionally found on lipid bound saccharide intermediates, but most often on completed repeat units (Tolmasky *et al.*, 1982). In polysaccharide producing strains of *Escherichia coli*, the oligosaccharide

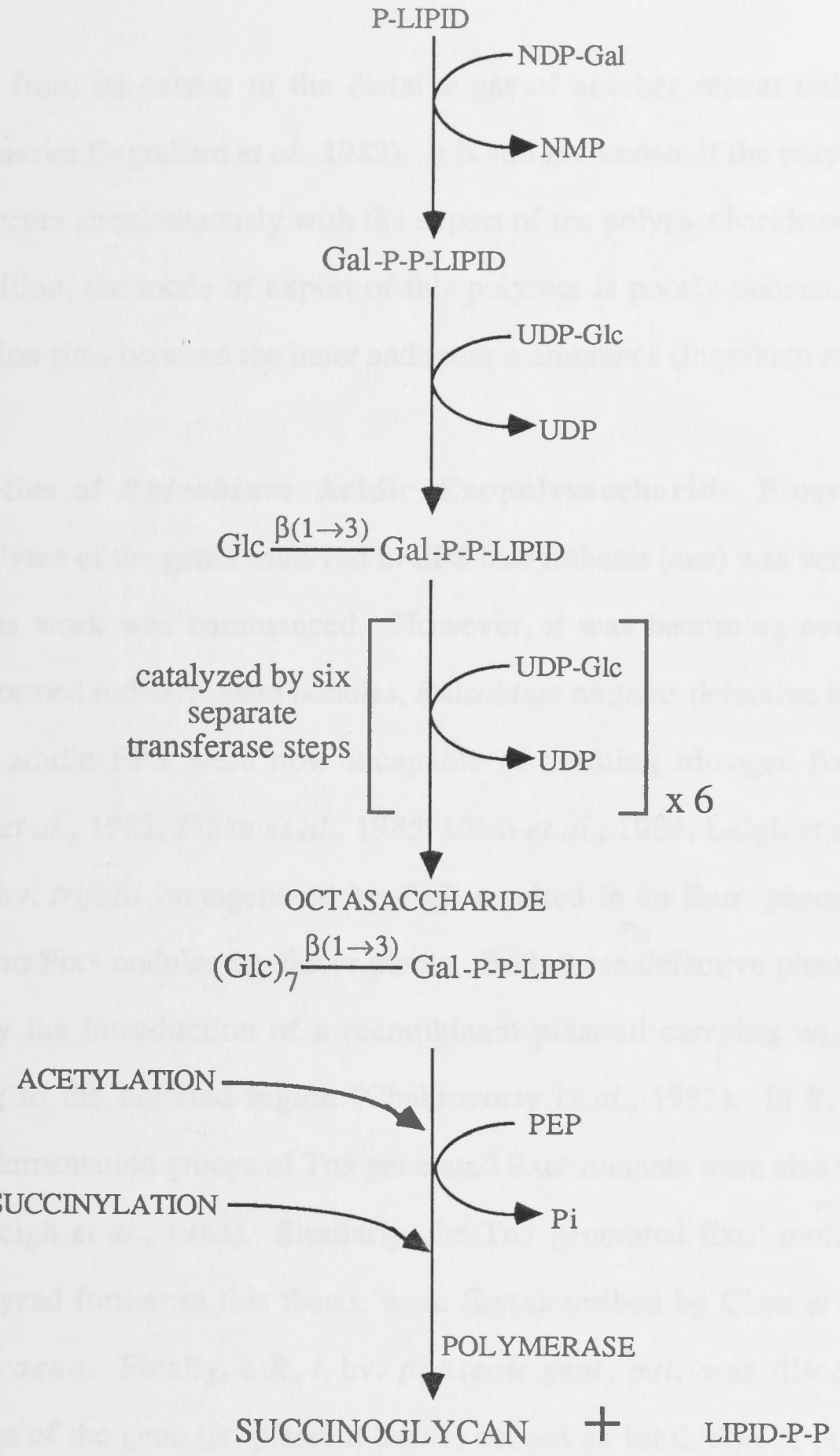


Fig. 1.4 Proposed pathway for the synthesis of succinoglycan (Tolmasky *et al.*, 1980 and 1982). Abbreviations: Gal, galactose; Glc, glucose; P, phosphate; PEP, phosphoenolpyruvate; NDP, nucleoside diphosphate; UDP, uridine diphosphate.

is transferred from its carrier to the distal sugar of another repeat unit on another phospholipid carrier (Ingraham *et al.*, 1983). It is still not known if the polymerization of repeat units occurs simultaneously with the export of the polysaccharide or prior to this event. In addition, the mode of export of this polymer is poorly understood, but may occur at adhesion sites between the inner and outer membranes (Ingraham *et al.*, 1983).

1.11 Genetics of *Rhizobium* Acidic Exopolysaccharide Biosynthesis

Molecular analyses of the genes involved in EPS biosynthesis (*exo*) was very scant at the time when this work was commenced. However, it was becoming evident that on legumes that formed indeterminate nodules, *Rhizobium* mutants defective in their ability to synthesize acidic EPS were now incapable of forming nitrogen-fixing nodules (Chakravorty *et al.*, 1982; Finan *et al.*, 1985; Chen *et al.*, 1985; Leigh *et al.*, 1985). A locus in *R. l.* bv. *trifolii* mutagenized by Tn*5*, resulted in an Exo$^-$ phenotype and an inability to form Fix$^+$ nodules on clover plants. Both these defective phenotypes could be restored by the introduction of a recombinant plasmid carrying wild-type DNA corresponding to the mutated region (Chakravorty *et al.*, 1982). In *R. meliloti*, six separate complementation groups of Tn*5* generated Exo$^-$ mutants were also symbiotically ineffective (Leigh *et al.*, 1985). Similarly, the Tn*5* generated Exo$^-$ mutants of *R.* sp. NGR234 analyzed further in this thesis, were first described by Chen *et al.* (1985) as Nod$^-$ on *Leucaena*. Finally, a *R. l.* bv. *phaseoli* gene, *psi*, was discovered when multiple copies of the gene (*ie.* plasmid borne) caused an inhibition of polysaccharide production by wild-type transconjugants carrying the allele (Borthakur *et al.*, 1985).

A great deal of progress has been made with the genetics of *Rhizobium* EPS biosynthesis over the last four years. *R. meliloti* has a cluster of twelve genes (see below) on the second megaplasmid, pRmeSU47b (Finan *et al.*, 1986; Keller *et al.*, 1988; Long *et al.*, 1988), and still more *exo* genes (*exoC*, *exoD*, *exoR* and *exoS*) on the chromosome (Finan *et al.*, 1986). Transposon insertion mutations within these various *exo* genes

affect EPS production and cause a variety of alfalfa infection deficiencies. Mutations in nine of the twelve pRmeSU47b borne *exo* genes either abolish or severely reduce EPS synthesis (Long *et al.*, 1988). Seven of these nine mutants (*exoP*, *exoM*, *exoA*, *exoL*, *exoF*, *exoQ* and *exoB*) form Fix$^-$ nodules, while the other two mutants (*exoJ* and *exoG*) induce Fix$^+$ nodules with a reduced efficiency. Mutations within *exoH* affect succinyl acetylation of the EPS and result in mutants that give Fix$^-$ nodules. Finally, mutations at another two *exo* genes (*exoN* and *exoK*) have no visual nodulation defect, despite the fact that they produce less EPS with one of them (*exoK*) delayed in its EPS production (Long *et al.*, 1988).

In addition to succinoglycan, a second less abundant EPS, termed EPS-II (Glazebrook and Walker, 1989) or EPSb (Zhan *et al.*, 1989), is also synthesized by *R. meliloti*. Synthesis of this second exopolysaccharide involves two genes from the succinoglycan pathway, *exoB* and *exoC*, and at least six of its own biosynthetic genes (*expA*, *expF*, *expC*, *expG*, *expD* and *expE*) also located on pRmeSU47b, and was regulated by a chromosomal locus (*expR* or *mucR*).

Three EPS regulatory genes (*psi, pss* and *psr*) from *R. l.* bv. *phaseoli* have been characterized in some detail, including DNA sequencing of *psi* and *pss* (Borthakur and Johnston, 1987; Borthakur *et al.*, 1985, 1986 and 1988). *R. l.* bv. *phaseoli* strains with a *psi*::Tn*5* mutation are not affected with regard to EPS production, but they are nonetheless, unable to form nitrogen-fixing nodules on *Phaseolus* beans. In addition, EPS synthesis in *R. l.* bv. *phaseoli* and *R. l.* bv. *viciae* was inhibited when the copy number of the wild-type *psi* allele was elevated (Borthakur *et al.*, 1985), but this inhibitory effect is counteracted by two other genes, *pss* and *psr* (Borthakur and Johnston, 1987; Borthakur *et al.*, 1988). The mode of *psi* suppression by *psr* occurs at the transcriptional level and is only partial. The suppression of *psi* by *pss* is reliant on the gene dosage of *pss* being elevated equally and the mode of its suppression is not at the

transcriptional level. The *pss* operon has an open reading frame (ORF) that encodes a putative protein of 200 amino acids and *psi* encodes a deduced 86 amino acid polypeptide. A Tn*5* mutation in the *pss* operon causes an Exo$^-$ phenotype and prevents nodulation of peas by *R. l.* bv. *viciae*, but does not affect symbiosis of beans with *R. l.* bv. *phaseoli*. EPS production by *psr*::Tn*5* mutants of *R. l.* bv. *phaseoli* is slightly reduced, but they are still symbiotically effective on beans (Borthakur and Johnston, 1987).

Additional exopolysaccharide genes which result in an over-production of EPS (Exo^{++}) when mutated by Tn*5*, have been found (Chen *et al.*, 1985; Doherty *et al.*, 1988; Müller *et al.*, 1988). These mutants probably represent inactivated negative regulatory loci. Strain ANU2895 is an Exo^{++} mutant of *R.* sp. NGR234, which was unable to form nitrogen-fixing nodules on *Leucaena*, but its nodulation of other hosts was not affected (Chen *et al.*, 1985). Similarly, there are some examples of Exo^{++} mutants of *R. meliloti*, but their Exo phenotypes did not always correlate with an inability to form nitrogen fixing nodules (Doherty *et al.*, 1988; Müller *et al.*, 1988). It seemed that the more severe cases of EPS deregulation resulted in more dramatic symbiotic deficiencies (Doherty *et al.*, 1988) and where the EPS structure was no longer the same as that of the wild-type, no nitrogen-fixing nodules would develop (Müller *et al.*, 1988). Two chromosomal genes that when mutated, result in an Exo^{++} phenotype in *R. meliloti*, termed *exoR* and *exoS*, were also shown to repress transcription of two pRmeSU47b located genes, *exoF* and *exoP*, which are essential for EPS biosynthesis (Doherty *et al.*, 1988). Furthermore, evidence suggested that expression of *exoR* may be affected by ammonia availability, which makes it a possible regulatory link with the environment (Doherty *et al.*, 1988).

1.12 Regulation of *Rhizobium* Exopolysaccharide Production During Symbiosis

The transformation of *Rhizobium* from free-living to the bacteroid form is marked by a number of morphological, physiological and biochemical changes. Bacteroids cease cell division, are much larger, show changes in cytochrome composition and most importantly fix atmospheric nitrogen (reviewed by Bergersen, 1982). The bacteroids also become enclosed within a membrane of plant origin (Paau *et al.*, 1978), which has the potential to regulate nutrient exchanges between bacteroid and host. The peribacteroid membrane is readily permeable to dicarboxylic acids, but is poorly permeable to hexose sugars (Price *et al.*, 1987).

Coordinated regulation of *exo* and *nod* genes has been demonstrated in *R. meliloti* through the activity of genes *syrM, syrA* and *nodD3* (Long *et al.*, 1989; Mulligan and Long, 1989). The *syrM* gene can increase the expression of *nod* genes in conjunction with *nodD3* and can also increase expression or activity of *exo* genes in conjunction with another locus, *syrA*. However, the involvement of *syrM* during symbiosis is less well defined, since mutations within *syrM* do not affect *in situ* expression of *nod* genes (Sharma and Signer, 1990).

Thus far, no specific polysaccharide genes have been shown to be expressed differently when the *Rhizobium* is in the bacteroid state as opposed to the free-living state. Fusions of *E. coli lacZ* to two neighboring transcriptional units involved in EPS production by *R. meliloti* were used to investigate possible regulation of these genes in the symbiotic state (Keller *et al.*, 1988). Transposon insertions into these transcriptional units eliminated the ability of cells to synthesize EPS and to effectively nodulate alfalfa. One of these loci (region III) corresponds to *exoF* by comparison of restriction maps (Keller *et al.* 1988) and hence may then be the same as *exoY* (described in this thesis). Transcriptional activity from these *exo* promoters was high in free-living rhizobia and

was still high when β-galactosidase was measured from bacteroids isolated from functional nodules. Whether or not the time taken to strip the bacteroids free of their insulating nodule tissue and measuring the activity of cloned promoters is sufficient to derepress a promoter, was not addressed.

The antigenicity of LPS from *R. l.* bv. *viciae* was shown to vary as the cells differentiate from the free-living form to the bacteroid form (VandenBosch *et al.*, 1989). An LPS antigen found on the surface of *R. l.* bv. *viciae* bacteroids, but not on the surface of free-living cells, was only expressed by free-living cells when cultures were grown at neutral pH with oxygen concentrations below 7.5% in the gas phase, or when cultures were grown aerobically at a pH less than 5.3 (Kannenberg and Brewin, 1989). The low oxygen conditions of the nodule (Tjepkema and Yocum, 1974) may therefore, be the environmental stimulus, which activates the developmental LPS gene(s) involved. Another possibility is acidity, but little is known about the pH of the peribacteroid units. Kannenberg and Brewin, (1989) suggest that it might be acidic due to the proton-ATPases and transport systems for dicarboxylic acids (Blumwald *et al.*, 1985, Udvardi *et al.*, 1988a), but they also discuss that the uptake of succinate and excretion of NH_3 would tend to raise the pH within the peribacteroid space. Whether the pH effect is relevant or not is unclear.

To date there have been no definitive results that can resolve the questions about regulation of exopolysaccharide synthesis during symbiosis. Certainly, the rate of EPS production by cultured bacteria varies over a wide range and is regulated by the growth conditions (*eg*. Jarman and Pace, 1984; Williams and Wimpenny, 1978; Souw and Demain, 1979). Industrial yields of polysaccharides are maximized by usually restricting cell division and protein synthesis by limiting the availability of usable nitrogen or sulfur sources, while simultaneously encouraging EPS production by providing an abundant supply of carbon source in the growth medium. Elucidation of possible regulatory

systems affecting EPS production by rhizobia during symbiosis would be facilitated by: (i) knowledge of the physical factors that effect its production by free-living rhizobia, (ii) knowledge of the physical environmental conditions that exists within nodule tissue, and (iii) electron-microscopical examination of nodule ultrastructures with particular attention to the presence or absence of polysaccharides. A comprehensive review of the literature for experiments of these types, is presented in the introduction to chapter 5.

1.13 *Rhizobium* sp. Strain NGR234

R. sp. NGR234 was isolated in Papua New Guinea from nodules on the roots of the cowpea-group fodder legume *Lablab purpureus* (L.) Sweet (Trinick, 1980). It was the only fast growing *Rhizobium* isolate capable of forming nitrogen-fixing nodules on *Lablab* and has since been shown to have a very broad host-range, which is uncharacteristic for fast growing *Rhizobium*. *R*. sp. NGR234 is known to nodulate at least 15 plant genera; including members of the Mimosideae sub-family as well as many members of the sub-family Papilionoideae, which is normally nodulated exclusively by slow growing *Bradyrhizobium* species. In addition, *R*. sp. NGR234 is unusual in that it is capable of nodulation, albeit not nitrogen fixation, on the root system of the non-legume tropical tree *Parasponia andersonii* (Trinick and Galbraith, 1980), which is a woody member of the Ulmaceae plant family. The broad host-range of *R*. sp. NGR234 makes it an interesting *Rhizobium* for the genetic analysis of determinants that govern host-range. Another fast growing, broad host-range rhizobia is strain MPIK3030 (Bachem *et al*., 1986), which is often referred to as a derivative of *R*. sp. NGR234 and while they are both similar, there are a number of properties which indicate that they are not identical.

1.14 Thesis Aims and Objectives

The possibility that exopolysaccharides on the surface of *Rhizobium* cells form part of a chemical-based recognition system between symbiotic partners, has gained considerable

support in recent years; and during the term of this study has also received much investigative attention. The aim of this study was to undertake a molecular analysis of the genetics and regulation of acidic exopolysaccharide biosynthesis in *Rhizobium* sp. strain NGR234 and to further investigate possible biological roles for the EPS. Mutants defective in their production of EPS had already been isolated and their symbiotic defects characterized for *R.* sp. NGR234 (Chen *et al.*, 1985) and for other *Rhizobium* species (see 1.11), but knowledge regarding the organization of these *exo* genes, their biosynthetic activity or regulation was not available.

One of the specific aims of this thesis was to conduct a molecular analysis of a region of wild-type *R.* sp. NGR234 DNA known to encode several *exo* genes. It was considered important to map loci in this region that affect EPS biosynthesis and to characterize genes at the DNA sequence level that were shown to have regulatory properties. Detailed information concerning the structure of such genes, when considered in conjunction with their resultant phenotypes in cultured and symbiotic *Rhizobium*, would thus provide information about their possible modes of action. Of interest also were other *trans* acting loci and physical factors that affect expression of these regulatory genes and attempts were made to mimic, *in vitro*, some of the conditions that might be experienced by the *Rhizobium* within nodule tissue.

Another aspect under investigation in this thesis was the role of EPS in nodule development and as a determinant of host-range. This was approached by first, examining the prevalence of the particular *exo* region across the *Rhizobium* genus and other EPS producing soil bacteria and then constructing hybrid strains for this region and analyzing their symbiotic abilities. The hybrid constructs also provided tremendous insight into the likely biochemical roles of the *exo* gene products in acidic exopolysaccharide biosynthesis.

CHAPTER TWO

Materials and Methods

2.1 MATERIALS

2.1.1 Bacterial Strains

Table 2.1 Bacterial strains used in this study

Strain	Description	Reference or source
Rhizobium sp. strain NGR234 derivatives		
NGR234	Wild-type broad-host-range cowpea *Rhizobium* sp.	Trinick 1980
ANU240	Sm^r derivative of NGR234	Morrison *et al.*, 1983
ANU265	Sm^r, Sp^r, pSym cured derivative of NGR234.	Morrison *et al.*, 1983
ANU280	Sm^r, Rif^r derivative of ANU240	Chen *et al.*, 1985
ANU1255	NGR234 *nodD*::Tn*5*.	Morrison *et al.*, 1984
ANU2807	ANU280 *exoY07*::Tn*5*	Chen *et al.*, 1988
ANU2808	ANU280 *exoY08*::Tn*5*	Chen *et al.*, 1988
ANU2811	ANU280 *exoY11*::Tn*5*	Chen *et al.*, 1988
ANU2818	ANU280 *exo* group G::Tn*5*	Chen *et al.*, 1988
ANU2820	ANU280 *exo* group A::Tn*5*	Chen *et al.*, 1988
ANU2822	ANU280 *exo* group C::Tn*5*	Chen *et al.*, 1988
ANU2823	ANU280 *exoY23*::Tn*5*	Chen *et al.*, 1988
ANU2824	ANU280 *exo* group C::Tn*5*	Chen *et al.*, 1988
ANU2826	ANU280 *exo* group B::Tn*5*	Chen *et al.*, 1988
ANU2831	ANU280 *exo* group G::Tn*5*	Chen *et al.*, 1988
ANU2833	ANU280 *exo* class 9::Tn*5*	Chen *et al.*, 1985
ANU2840	ANU280 *exoY40*::Tn*5*	Chen *et al.*, 1988
ANU2841	ANU280 *exoY41*::Tn*5*	Chen *et al.*, 1988
ANU2842	ANU280 *exoY42*::Tn*5*	Chen *et al.*, 1988
ANU2844	ANU280 *exoY44*::Tn*5*	Chen *et al.*, 1988
ANU2845	ANU280 *exoY45*::Tn*5*	Chen *et al.*, 1988
ANU2847	ANU280 *exoY47*::Tn*5*	Chen *et al.*, 1988
ANU2851	ANU280 *exoY51*::Tn*5*	Chen *et al.*, 1988
ANU2852	ANU280 *exoY52*::Tn*5*	Chen *et al.*, 1988
ANU2854	ANU280 *exoY54*::Tn*5*	Chen *et al.*, 1988
ANU2861	ANU280 *exo* class 9::Tn*5*	Chen *et al.*, 1985
ANU2864	ANU280 *exoY64*::Tn*5*	Chen *et al.*, 1988
ANU2865	ANU280 *exoY65*::Tn*5*	Chen *et al.*, 1988
ANU2866	ANU280 *exo* class 9::Tn*5*	Chen *et al.*, 1985
ANU2867	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988

Table 2.1 Continued

Strain	Description	Reference or source
ANU2871	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988
ANU2872	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988
ANU2873	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988
ANU2876	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988
ANU2877	ANU280 *exo* group D::Tn*5*	Chen *et al.*, 1988
ANU2890	ANU280 *exoY90*::Tn*5*	Chen *et al.*, 1988
ANU2895	ANU280 *exo* class 9::Tn*5*	Chen *et al.*, 1985
616-d1	Km^s, ANU2811 16 kb *exo* deletion	This work
616-d2	Km^s, ANU2811 16 kb *exo* deletion	This work
616-d3	Km^s, ANU2811 16 kb *exo* deletion	This work
616-d4	Km^s, ANU2811 16 kb *exo* deletion	This work
Other *Rhizobium* and *Agrobacterium* species and strains		
C58	Wild-type *Agrobacterium tumefaciens*	Casse *et al.*, 1979
GMI9023	C58 derivative, cured of pAtC58 and pTiC58	Rosenberg and Huguet, 1984
ANU843	Wild-type *R. l.* bv. *trifolii*	Djordjevic *et al.*, 1983
ANU845	pSym cured derivative of ANU843	Schofield *et al.*, 1984
Rm1021	Sm^r derivative of wild-type *R. meliloti*	Meade *et al.*, 1982
Rm7013	Rm1021 *exoB13*::Tn*5*	Leigh *et al.*, 1985
Rm7031	Rm1021 *exoA31*::Tn*5*	Leigh *et al.*, 1985
Rm7055	Rm1021 *exoF55*::Tn*5*	Leigh *et al.*, 1985
USDA191	Wild-type *R. fredii*	Appelbaum *et al.*, 1985
Escherichia coli strains		
HB101	*leu proA2 rps120* (Sm^r) *hsdS20*	Bolivar *et al.*, 1977
JM107	Δ(*lac-proAB*) *thi, supE44 endA1 hsdR17*(r^-m^+) *gyrA96 relA1* [F'*traD36 proAB lacI*^{q} *ZΔM15*]	Yannisch-Perron *et al.*, 1985
NM522	$recA^+$, (*supE*, *thi*, Δ(*lac-proAB*), *hsd5*, {F', *proAB*, *lacI*^{q}, *lacZΔM15*})	Gough and Murray, 1983
POII1734	MC1040 (mu *cts*) with Mu dII-1734 *lac*$^+$	Castilho *et al.*, 1984

2.1.2 Plasmids, Cosmids, Phagemids and Bacteriophages

Table 2.2 Plasmids, cosmids, phagemids and bacteriophages used in this study

Plasmid	Description	Reference or source
Vectors, helper plasmids/bacteriophages and non-*exo* clones		
Bluescribe	*E. coli* specific, *lac'*Z, Ap^r	Vector cloning systems San Diego, CA 92121, USA
Bluescript (SK+, SK-, KS+, KS-)	*E. coli* specific, Ap^r, F_1 phage ORI (+ or - strand), KS or SK polylinker	Stratagene, San Diego, CA 92121, USA.
M13mp18, 19	*E. coli* specific bacteriophage reversible polylinker	Norrander *et al.*, 1983
pBR322	*E. coli* specific, Tc^r, Ap^r.	Boliver *et al.*, 1977
pJJ016	Broad host-range, derived from pRK290 (Ditta *et al.*, 1980), Km^r, Tc^r.	Weinman, 1986
pKAN2	pBR322 recombinant carrying the 3.5 kb *Hin*dIII fragment of Tn*5*.	Scott *et al.*, 1982
pMP220	Broad host-range IncP1, Tra$^-$, promoterless *lacZ*, Tc^r.	Spaink *et al.*, 1987
pRK2013	Helper plasmid, Tra$^+$, *oriT*, ColE1, Km^r.	Ditta *et al.*, 1980
pSUP106	Broad host-range IncQ, Tra$^-$, Tc^r, Cm^r.	Simon *et al.*, 1983
pUC18	*E. coli* specific, *lac'*Z, Ap^r	Vierra and Messing, 1982
VCSM13	Km^r, helper phage for ssDNA isolation from bluescript recombinants	Stratagene, San Diego, CA 92121, USA.
Recombinant plasmids carrying *R.* sp. NGR234 DNA		
pARI7	pMP220 recombinant, wild-type *Eco*RI fragment corresponding to 2895::Tn*5* region	Tony Arioli, ANU, Canberra, Australia.
pHC11	pBR322 recombinant carrying the Tn*5* mutated *Eco*RI fragment of ANU2811.	Chen *et al.*, 1988
pHC20	pMP220 recombinant carrying the Tn*5* mutated *EcoRI* fragment of ANU2820.	Hancai Chen, ANU, Canberra, Australia.
pHC26	pMP220 recombinant carrying the Tn*5* mutated *EcoRI* fragment of ANU2826.	Hancai Chen, ANU, Canberra, Australia.
pHC67	Bluescribe recombinant carrying the Tn*5* mutated *EcoRI* fragment of ANU2867.	Hancai Chen, ANU, Canberra, Australia.

Table 2.2 Continued

Strain	Description	Reference or source
pHC71	Bluescribe recombinant carrying the Tn*5* mutated *EcoRI* fragment of ANU2871.	Hancai Chen, ANU, Canberra, Australia.
pJG11	Ap^r, 10 kb *Bam*HI fragment of wild-type *exo* DNA, cloned into pUC18	This work
pJG20	pJJ016 recombinant carrying the same insert as pJG11, Tc^r.	This work
pJG22	Insert in opposite orientation to pJG20	
pJG22::Tn*5*	Same clone as pJG22, but with a Tn*5* at the *exoY11* position, Tc^r, Km^r.	This work
pJG30	Bluescribe recombinant carrying an 8 kb *Eco*RI fragment of wild-type *exo* DNA.	This work
pJG40	pUC18 recombinant carrying a 9 kb *Bam*HI fragment of wild-type *exo* DNA	This work
pJG51*	7.0kb *Bgl*II subclone	This work
pJG52*	5.0kb *Pst*I subclone	This work
pJG53*	1.9kb *Cla*I-*Pst*I subclone	This work
pJG54*	1408bp *Mlu*I-*Pst*I subclone *exoY'-lacZ*$^+$	This work
pJG55*	1221bp *Mlu*I-*Hind*III partial subclone	This work
pJG56*	992bp *Mlu*I-*Nru*I partial subclone	This work
pJG57*	790bp *Mlu*I-*Eco*RI subclone	This work
pJG58*	1121bp *Nru*I-*Pst*I partial subclone	This work
pJG59*	934bp *Nru*I-*Hind*III partial subclone	This work
pJG60*	705bp *Nru*I subclone *exoX'-lacZ*$^+$	This work
pJG61*	923bp *Hind*III-*Pst*I partial subclone	This work
pJG62*	736bp *Hind*III subclone	This work
pJG63*	630bp *Eco*RI subclone	This work
pJG64*	695bp *Hind*III subclone	This work
pJG65*	1392bp *Eco*RI subclone	This work
pJG66*	887bp *Nru*I-*Cla*I partial subclone	This work
pJG70*	the pJG60 insert in opposite orientation	This work
pJG100	pSUP106 recombinant carrying the insert as pJG11, Cm^r.	This work

* These plasmids carry subcloned regions of the pJG11 insert DNA cloned into the vector pMP220 and are represented in Fig. 4.8

Table 2.2 Continued

Strain	Description	Reference or source
R'2811	pMN2 recombinant carrying ~70 kb of DNA flanking *exoY11*::Tn*5* allele, Tc^r, Km^r.	Chen *et al.*, 1988
R'3222	Tc^r, pMN2 recombinant carrying ~65 kb of wild-type *exo* DNA	Chen *et al.*, 1988
Recombinant plasmids carrying other *Rhizobium exo* DNA		
pD2	*R. meliloti exoB* complementing cosmid	Leigh *et al.*, 1985
pD15	*R. meliloti exoC* complementing cosmid	Leigh *et al.*, 1985
pD5	*R. meliloti exoD* complementing cosmid	Leigh *et al.*, 1985
pD34	*R. meliloti exoA* complementing cosmid	Leigh *et al.*, 1985
pD56	*R. meliloti exoBF* complementing cosmid	John Leigh, Washington State Univ.
pD56-306	pD56 with a Tn*5* insertion at *exoF306*	Long *et al.*, 1988
pD56-321	pD56 with a Tn*5* insertion at *exoG321*	Long *et al.*, 1988
pD56-332	pD56 with a Tn*5* insertion at *exoQ332*	Long *et al.*, 1988
pD56-347	pD56 with a Tn*5* insertion at *exoB347*	Long *et al.*, 1988
pRG100	Wild-type *R. meliloti exo* DNA, Tc^r.	Hangjun Zhan, Washington State University
pRK14-17	Wild-type *nodD2* clone from USDA191 in pRK290 (Ditta *et al.*, 1980)	Appelbaum *et al.*, 1988
pRK14-17K1	*nodD2*::Tn*5* mutant allele in pRK290	Appelbaum *et al.*, 1988

2.1.3 Plant Species

Table 2.3 Plant varieties used in this study

Plant species	Cultivar	Common name
Leucaena leucocephala	(Lam.) Wit. var. Peru	*Leucaena*
Macroptilium atropurpureum	Urban	siratro
Medicago sativa	Hunter river	alfalfa or lucerne

2.1.4 Bacterial Culture Media

(i) Gamborg's trace element solution (Gamborg and Eveleigh, 1968)

Nutrient	Concentration of stock solution	Final concentration from a 10^{-3} dilution
$MnSO_4.4H_2O$	10.0 $g.l^{-1}$	45 µM
H_3BO_3	3.0 $g.l^{-1}$	48 µM
$ZnSO_4.7H_2O$	3.0 $g.l^{-1}$	10 µM
$Na_2MoO_4.2H_2O$	0.25 $g.l^{-1}$	1.0 µM
$CuSO_4.5H_2O$	0.25 $g.l^{-1}$	1.0 µM
$CoCl_2.6H_2O$	0.25 $g.l^{-1}$	1.0 µM

(ii) BMM: Bergersen's modified medium (Bergersen, 1961)

This was used as a general purpose *Rhizobium* growth medium and was particularly useful for visualizing Exo phenotypes.

Nutrient	Concentration of stock solution	Amount added to 1000 ml of medium
$Na_2HPO_4.12H_2O$	45 $g.l^{-1}$	8.0 ml
$MgSO_4.7H_2O$	10 $g.l^{-1}$	8.0 ml
$FeCl_3$	20 $g.l^{-1}$	0.15 ml
$CaCl_2.2H_2O$	40 $g.l^{-1}$	1.0 ml
Gamborg's trace elements		1.0 ml
Thiamine	2.0 $g.l^{-1}$	1.0 ml
Biotin	0.2 $g.l^{-1}$	1.0 ml
Monosodium glutamate	-	0.5 g
Mannitol	-	3.0 g
Yeast extract	-	0.5 g
Agar* (Difco)	-	15 g

pH = 7.0

* added to solid medium only

(iii) MX: Mops media

This is a strongly buffered and absolutely defined media for the growth of *Rhizobium* under strictly controlled growth conditions.

Nutrient	Concentration of stock solution	Amount added to 1000 ml of medium
Morpholinepropane-sulfonic acid (MOPS)	-	10.5 g
$CaCl_2.2H_2O$	150 $g.l^{-1}$	1.0 ml
Gamborg's trace elements	-	1.0 ml
TM vitamins	-	2.5 ml
Fe-chelate solution	-	1.0 ml
$FeSO_4.7H_2O$	2.8 $g.l^{-1}$	
Na_2-EDTA	3.72 $g.l^{-1}$	
N-B5 salts	-	100 ml
$NaH_2PO_4.2H_2O$	1.5 $g.l^{-1}$	
KI	7.5 $mg.l^{-1}$	
KCl	5.0 $g.l^{-1}$	
Na_2SO_4	1.5 $g.l^{-1}$	
$MgSO_4.7H_2O$	2.5 $g.l^{-1}$	
Nitrogen source*	-	7.5 mM
Carbon source¥	-	40 mM
Monosodium glutamate†	-	10 mM

pH = 7.0 (with 5 M KOH)

* Depending upon the nature of the experiment, a range of $[NH_4^+]$ and $[NO_3^-]$ were used in the forms of either NH_4Cl, $(NH_4)_2SO_4$, KNO_3 or NH_4NO_3. When nitrogen conditions were not being tested, the standard was NH_4NO_3 at 0.6 $g.l^{-1}$.

¥ Depending upon the nature of the experiment, a range of carbon sources were examined, either separately or in combinations, made up to a total concentration of 40 mM.

† Monosodium glutamate can serve as a carbon source and as a nitrogen source; thus, when these parameters were being tested, its use in the medium was governed by the nature of the experiment.

(iv) TMR: Modified *trifolii* medium (Innes *et al.*, 1985)

This medium supports the growth of *Rhizobium* but not of *E. coli* and is therefore useful when such a selection is desired.

Nutrient	Concentration of stock solution	Amount added to 1000 ml of medium
$MgSO_4.7H_2O$	100 $g.l^{-1}$	2.0 ml
$FeCl_3$	3.0 $g.l^{-1}$	1.0 ml
$CaCl_2.2H_2O$	50 $g.l^{-1}$	1.0 ml
Sucrose	-	10.0 g
Gamborg's trace elements	-	1.0 ml
TMR salts		50 ml
K_2HPO_4	20.8 $g.l^{-1}$	
KH_2PO_4	8.8 $g.l^{-1}$	
NaCl	2.0 $g.l^{-1}$	
$(NH_4)_2SO_4$	10.0 $g.l^{-1}$	
TM vitamins		2.5 ml
Thiamine	0.4 $g.l^{-1}$	
Biotin	0.2 $g.l^{-1}$	
Pyridoxine	0.4 $g.l^{-1}$	
Nicotinic acid	0.4 $g.l^{-1}$	
Agar	-	15 g

pH = 7.0

(v) TY: Tryptone yeast medium (Beringer, 1974)

Rhizobium produces less exopolysaccharide when cultured on this medium and it is therefore useful when cells are being cultured for DNA isolations or transconjugation.

Nutrient	Amount added to 1000 ml of medium
Bacto-tryptone	5.0 g
Yeast extract	3.0 g
$CaCl_2.2H_2O$ †	0.9 g
Agar* (Difco)	15 g

pH = 7.0

† added separately when at room temperature

* added to solid medium only

(vi) LB: Luria Broth medium (Miller, 1972)

This was a general purpose medium for the culture of *E. coli*, but will not support growth of *R.* sp. NGR234.

Nutrient	Amount added to 1000 ml of medium
Bacto-tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g
glucose	1.0 g
Agar* (Difco)	15 g

pH = 7.0

* added to solid medium only

2.1.5 Antibiotic Concentrations

The following antibiotics and their concentrations were used for relevant *Rhizobium* and *E. coli* strains.

Antibiotic	Concentrations for *Rhizobium*	Concentrations for *E. coli*
Ampicillin (Ap)	-	100 $\mu g.ml^{-1}$
Chloramphenicol (Cm)	80 $\mu g.ml^{-1}$	30 $\mu g.ml^{-1}$
Kanamycin (Km)	200 $\mu g.ml^{-1}$	50 $\mu g.ml^{-1}$
Rifampicin (Rif)	30 $\mu g.ml^{-1}$	-
Spectinomycin (Sp)	200 $\mu g.ml^{-1}$	-
Streptomycin (Sm)	400 $\mu g.ml^{-1}$	-
Tetracycline (Tc)	4 $\mu g.ml^{-1}$	15 $\mu g.ml^{-1}$

2.1.6 Plant Culture Media

(i) F: Fåhræus medium (Fåhræus, 1957)

F medium was used as a nitrogen free, solid medium for growth of *Leucaena*, siratro and alfalfa plants.

Nutrient	Concentration of stock solution	Final concentration	Amount added to 1000 ml of medium
$CaCl_2.2H_2O$	10.0 $g.l^{-1}$	0.7 mM	10 ml
$MgSO_4.7H_2O$	12.0 $g.l^{-1}$	0.5 mM	10 ml
KH_2PO_4	10.0 $g.l^{-1}$	0.7 mM	10 ml
$Na_2HPO_4.12H_2O$	15.0 $g.l^{-1}$	0.4 mM	10 ml
Fe-citrate	0.50 $g.l^{-1}$	0.02 mM	10 ml
Gamborg's trace elements	-	(see 2.1.4.i)	1.0 ml
Agar	-	-	15 g

pH = 6.5 (adjusted with NaOH)

(ii) Herridge's medium (Herridge, 1977).

Herridge's medium is nitrogen free and was used at half strength in liquid form, for *Leucaena leucocephala* plant assays grown in Magenta jars (see 2.2.5).

Nutrient	Concentration of stock solution	Final concentration	Volume to make up 10 litres of half strength
$CaCl_2.2H_2O$	145 $g.l^{-1}$	125 µM	1.25 ml
KCl	74.6 $g.l^{-1}$	125 µM	1.25 ml
$MgSO_4.7H_2O$	247 $g.l^{-1}$	250 µM	2.50 ml
KH_2PO_4	136 $g.l^{-1}$	65 µM	0.65 ml
K_2HPO_4	174 $g.l^{-1}$	65 µM	0.65 ml
Gamborg's trace elements	-	(see 2.1.4.i)	1.25 ml
FeNa-EDTA	345 $g.l^{-1}$	133 µM	1.25 ml

No pH adjustment required (pH = 6.5 - 7.0)

2.1.7 Oligodeoxynucleotides

The following oligodeoxynucleotide primers were used for DNA sequencing with the Bluescript vector system: T3 primer, T7 primer, SK primer and KS primer; they were all purchased from Stratagene, San Diego, CA 92121, USA. For DNA sequencing with the M13mp vector system the "17mer Sequencing Primer (-20) #1211" (New England Biolabs Catalog, 1986/87, p 129) was used; purchased from Bio-Rad Laboratories, Richmond, CA 94801, USA. For DNA sequencing of Tn*5* insertion sites, a specific Tn*5* primer was used (a gift from John Watson, CSIRO, Canberra, Australia).

Tn*5* primer

```
          5'-CGTTCAGGACGCTACTT-3'  →   Direction of polymerase
             :::::::::::::::::
.....CCAAGGCAAGTCCTGCGATGAACACATATTCTCAGTC-5'  Terminus
```

Terminal 37 bp of Tn*5* flanking repeat

2.2 Microbiological Techniques and Plant Nodulation Assays

2.2.1 *Rhizobium* Liquid Culture Growth Conditions

The important details of growth conditions for various *Rhizobium* liquid cultures through out this thesis (especially in chapter 5) are outlined in their relevant sections. In general, *Rhizobium* was cultured at 28°C, with vigorous shaking. A high level of turbulence, achieved by using baffled flasks with loosely fitting metal caps, enabled maximal aeration in the liquid cultures. In experiments where the gas phase within the flasks were to be sealed from the outside, rubber "Suba-Seal" stoppers (William Freeman & Company Ltd., Yorkshire, England) were used.

2.2.2 Storage of Bacterial Strains

(i) *Rhizobium* strains were stored for periods of 12 to 24 months at room temperature, as stabs in BMM media in small vials; or as suspensions in a solution of 10% sucrose and 12% glycerol, at -70°C.

(ii) *E. coli* strains were stored for several years at -20°C as a suspension in 33% glycerol and 67% LB media.

2.2.3 Bacterial Transconjugation

(i) Patch mating

This was a very simple and convenient method for the transfer of broad-host plasmids from *E. coli* into *Rhizobium* in order to examine the phenotypes of *Rhizobium* transconjugants. Self-transmissible plasmids, such as the R-prime plasmids used in this study, could be transferred simply by mixing donor and recipient strains. However, the transfer of non-self-transmissible plasmids required the *tra* genes carried on the helper plasmid, pRK2013 (Ditta *et al.*, 1980). The protocol for such patch matings was as follows. Donor *E. coli* strains were grown freshly on solid LB media and recipient *Rhizobium* strains were grown freshly on TY solid medium, as this reduced the amount of exopolysaccharide secreted and thereby improved the frequency of plasmid transfer.

A small clump of recipient *Rhizobium* cells on the end of a 15 cm tooth pick was spotted onto solid TY medium; a similar small clump of donor *E. coli* cells was spotted on top of the recipient cells; finally a similar amount of cells of the helper strain was placed on top of the donor and recipient cells and all three bacterial strains were mixed thoroughly with a stick. Control patches of the three strains spotted separately and in paired combinations were also set up on the same plate of TY media to detect any contamination between the parental strains. The cultures were incubated at 28°C for 16 hr, before being replica plated onto BMM or TMR plates that had been supplemented with appropriate antibiotics and incubated again until the transconjugants had grown. The resultant transconjugants were restreaked and purified until there was no doubt of donor contamination, before they were used in subsequent biological assays.

(ii) Filter mating

Recipient, donor and helper (when appropriate) strains were grown freshly on solid TY or LB media for *Rhizobium* or *E. coli* respectively. All strains were resuspended in liquid TY media to approximately equal densities and 2 ml aliquots of each parental strain were mixed in a test tube by vortexing. The mating mixture was filtered through a sterile 0.45 μm nitrocellulose filter (Millipore Inc., Detroit , USA.). The bacteria remained on the nitrocellulose disc which was placed bacterial side up on solid TY media and incubated at 28°C for 16 hr. A liquid suspension of the bacteria was prepared by placing the entire disc in a tube containing 4 ml of sterile water and vortexing. Serial dilutions of the bacterial solution were plated out to select for the transconjugants and to ascertain the frequency of donor, recipient and transconjugant cells in the mixture. Transconjugation frequencies could be presented as the ratio of transconjugants to total recipient cells.

2.2.4 Sterilization of Seeds

(i) *Leucaena leucocephala*

The seed coats were scarified by soaking the seeds in concentrated H_2SO_4 (10 M) for 7 min. and mixing continuously by inversion. The seeds were then thoroughly rinsed with tap water; and then soaked for 30 min. in full strength commercial grade sodium hypochlorite (12.5%) with continuous rotary shaking. The sodium hypochlorite was washed out of the seeds with seven, 2 min. washes with sterile, distilled water. The seeds were then placed on BMM agar plates and germinated for 36 hr in the dark at 28°C.

(ii) *Macroptilium atropurpureum* (siratro)

The seed coats were scarified by soaking the seeds in concentrated H_2SO_4 (10 M) for 5 min. without agitation. The seeds were then thoroughly rinsed with tap water and then soaked for 10 min. in 6.25% sodium hypochlorite (diluted to 50% of the commercial strength) without agitation. The sodium hypochlorite was washed out of the seeds with five, 2 min. washes with sterile, distilled water. The seeds were then placed on BMM agar plates and germinated for 24 hr in the dark at 28°C.

(iii) *Medicago sativa* (alfalfa)

The seeds were soaked in 100% ethanol for 15 min. and then rinsed with distilled water. Sterilization was by soaking the seeds in 6.25% sodium hypochlorite (diluted to 50% of the commercial strength) without agitation for 12 min. The sodium hypochlorite was washed out of the seeds with five, 2 min. washes with sterile, distilled water. The seeds were then placed on BMM agar plates and synchronous germination was achieved by incubation in the dark, firstly at 4°C for 24 hr. and then at 28°C for 24 hr.

2.2.5 Plant Nodulation Assays

(i) Magenta jar assay (*Leucaena*)

This was the quickest and most successful assay system for nodulation of *Leucaena*, presumably since the growth conditions more closely mimicked the natural environment; such as, continuous availability of moisture, roots maintained in darkness and the shoot exposed to the open air. The method was originally developed by E. R. Appelbaum as a variation of the Leonard Jar Assay (Vincent, 1970). Magenta jars were purchased from Magenta Corp., Chicago, USA, made of clear autoclavable plastic, are 76 x 76 x 103 mm and are slightly tapered at the base such that one jar can be placed partially inside another (Fig. 2.1). The upper jar was filled with vermiculite; the lower jar contained the half strength liquid Herridge's plant medium and a rope wick passed through a hole created in the base of the upper jar and descended to the inside surface of the lower jar's base (Fig. 2.1). The vermiculite layer was covered with a plastic lid and the whole system was autoclaved. After autoclaving, small germinated seedlings were planted into the vermiculite (4 seedlings per jar) and inoculated. The plants in the Magenta jar apparatus were then grown in cabinets with strictly controlled growth conditions (16 hr of light at an intensity of 350-400 $\mu E^{-2}.s^{-1}$, 80% humidity, the temperature in the vermiculite was 26°C in the light cycle and 22°C in the dark cycle). After one or two days, the lids were removed from the upper jar to give the seedlings more space and the top surface of the vermiculite was then covered with sterilized small pebbles or coarse sand to prevent contamination. During the course of the average four to six week experiment, the media reservoir would be depleted at least once and this was replenished by replacing the lower jar with another that was filled with sterile half strength Herridge's media. This practice minimized the possibilities of cross contamination that would otherwise readily occur if topping up of the reservoir from a common bottle was the procedure employed.

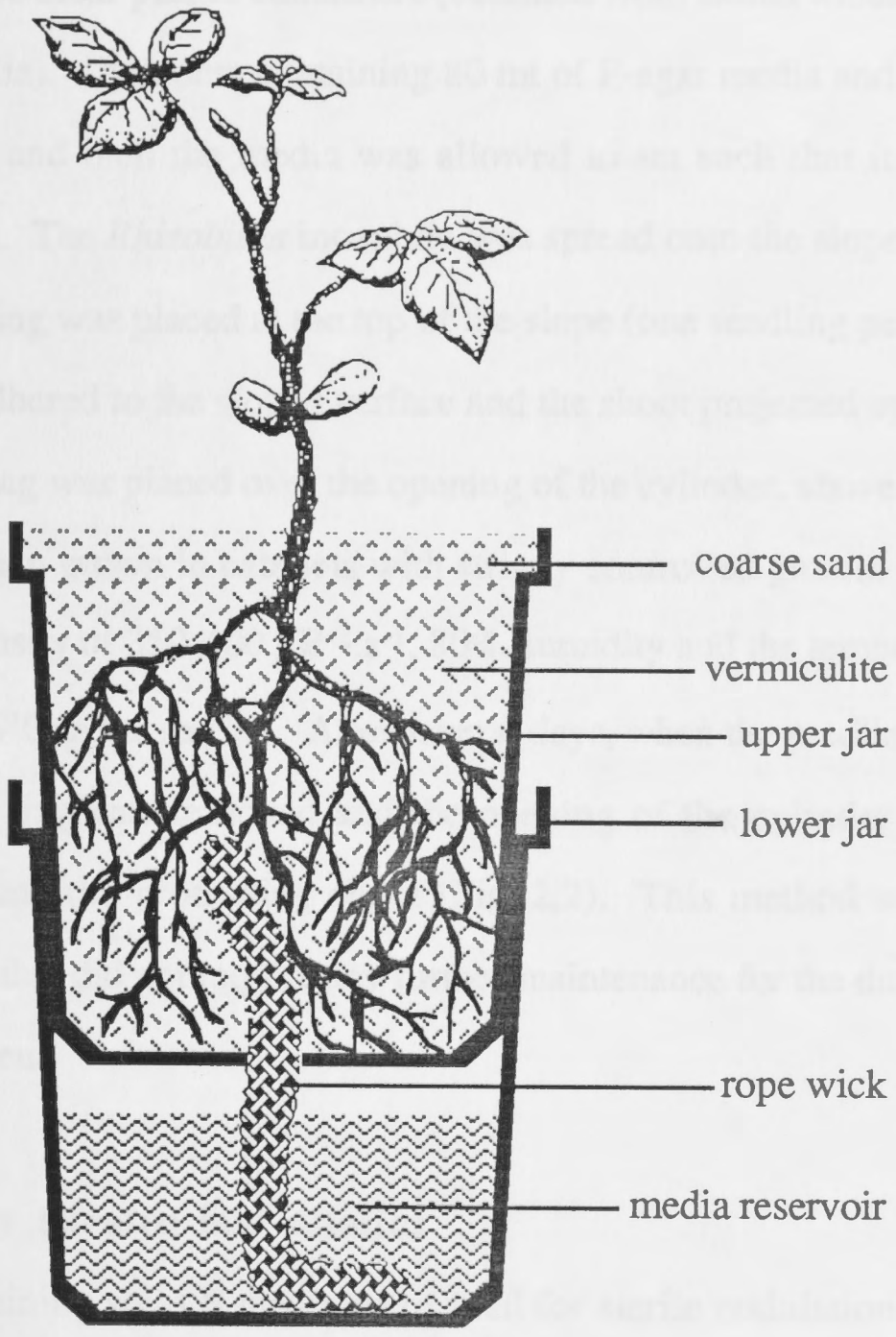


Fig.2.1 Magenta jar apparatus. This assay technique was the preferred system for *Leucaena* nodulation experiments. The upper jar could be partially inserted into the lower jar to form an air-tight seal. This figure was copied from one that appears in Brant J. Bassam's Ph.D. Thesis, 1988, Australian National University.

(ii) Plastic cylinders (*Leucaena*)

This method was used for *Leucaena* and allowed the ongoing development of nodule structures to be monitored and compared, because the root systems were continuously visible through the clear plastic containers (obtained from Bunzl Medical & Laboratory Products, Australia). Cylinders containing 80 ml of F-agar media and with a screw cap were autoclaved and then the media was allowed to set such that it formed a sloped surface (Fig. 2.2). The *Rhizobium* inoculum was spread onto the sloped surface and the germinated seedling was placed at the top of the slope (one seedling per container), such that the radical adhered to the sloped surface and the shoot projected upwards (Fig. 2.2). A sterile plastic bag was placed over the opening of the cylinder, above the seedling; and the plants were then grown in cabinets with strictly controlled growth conditions (16 hr of light at an intensity of 350-400 $\mu E^{-2}.s^{-1}$, 80% humidity and the temperature during the light cycle was 26°C in the media). After several days, when the seedling had established itself, the plastic bag was removed and the opening of the cylinder was sealed with Nescofilm® around the protruding shoot (Fig. 2.2). This method was a sealed self-contained system that did not require any further maintenance for the duration of a five or six week experiment.

(iii) Plate assay (siratro and alfalfa)

Petri dishes containing solid F media were used for sterile nodulation assays of siratro and alfalfa. The *Rhizobium* inoculum was spread onto the surface of the media and the germinated seedlings (two or three per plate) were placed in a row, one quarter from the edge of the plate. The plates were sealed with Nescofilm, which was pierced with a small hole to allow gas exchange; and they were then incubated on their edges in cabinets with strictly controlled growth conditions (16 hr of light at an intensity of ~400 $\mu E^{-2}.s^{-1}$ and the temperature during the light cycle was 22°C in the plate) for the duration of the nodulation experiment and without any further attention or maintenance.

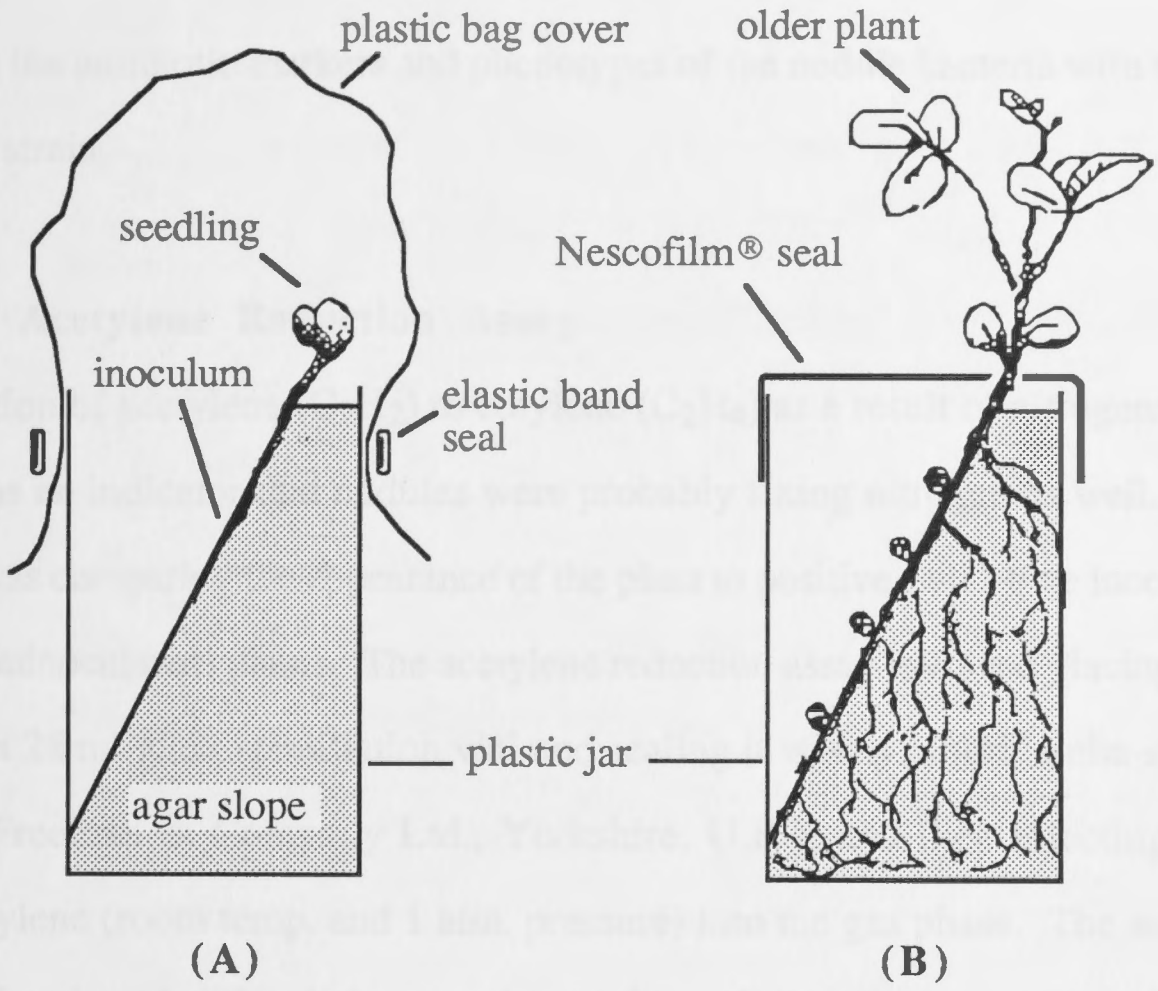


Fig. 2.2 Plastic cylinder apparatus. **(A).** The system is covered with a plastic bag for the first few days and **(B)** aseptic conditions are maintained on the root surface with Nescofilm after the plastic bag has been removed. This figure was copied from one that appears in Brant J. Bassam's Ph.D. Thesis, 1988, Australian National University.

2.2.6 Isolation of Nodule Bacteria

The following protocol is an adaptation of one reported by Gresshoff *et al.* (1977). Nodules were cut from the root, dipped into 100% ethanol and surface sterilized by submersing in a drop of 1.25% sodium hypochlorite (10% of commercial strength) for 10 min. The surface-sterilized nodules were then rinsed three times in separate drops of sterile distilled water and rolled over the surface of a solid BMM plate to verify the sterility of the nodule surface. The nodule was then transferred to a drop of protoplast dilution buffer (0.25 M sorbitol, 0.25 M mannitol, 2 mM $CaCl_2$) and crushed. The crushed nodule tissue was then streaked onto a BMM plate with extra mannitol at 6 $g.l^{-1}$. Genetic recombination events or the presence of contaminants could be detected by

comparing the antibiotic markers and phenotypes of the nodule bacteria with those of the inoculated strain.

2.2.7 Acetylene Reduction Assay

The reduction of acetylene (C_2H_2) to ethylene (C_2H_4) as a result of nitrogenase activity was used as an indicator that nodules were probably fixing nitrogen as well. The other indicator was comparing the appearance of the plant to positive (wild-type inoculated) and negative (uninoculated) plants. The acetylene reduction assay involved placing the whole plant into a 28 ml glass scintillation vial and sealing it with a rubber Suba-seal stopper (William Freeman & Company Ltd., Yorkshire, U.K.), and then injecting 1.0 ml of 100% acetylene (room temp. and 1 atm. pressure) into the gas phase. The sealed plants were then incubated under their normal growth conditions for approximately 5 hr. and then 200 µl samples were removed from the gas phase and analyzed for ethylene by hydrogen flame ionization gas chromatography using a Poropak R-80-100 column at 45°C with N_2 gas as the mobile phase. Quantification of ethylene was done by reference to calibrated standards.

2.2.8 Measurement of EPS

The amounts of polysaccharide produced by *Rhizobium* cultures was estimated using an anthrone–H_2SO_4 method, modified from that described by Seifter *et al.* (1950). Anthrone gives a blue colour reaction when condensed with dehydrated hexose sugars. Anthrone solution (0.2% anthrone in 98% H_2SO_4) was allowed to equilibrate for an hour before being used and was only considered reliable for 24 hours. Cultures were grown in liquid MX media using 20 mM Na–succinate and 20 mM Na–glutamate as carbon sources and and 10 mM NH_4Cl as the nitrogen source. It was important not to have NO_3^- ions in the media as these interfered with the anthrone reaction and a red compound was generated. The reaction was started by slowly adding 8 ml of anthrone solution to 4 ml of culture, that had been chilled on ice. The solution was mixed and immersed in a

92°C water bath for 8 min, which was in excess of the time required for the reaction to go to equilibrium. The reaction tubes were then kept on ice until the absorbances at λ=585 nm were determined. All estimations were performed with three or more repeats, zeroed against the media+anthrone solution and measured against a standard curve using glucose dissolved in media.

2.2.9 Measurement of Protein

The protein samples were prepared in eppendorf tubes, from 1ml samples of *Rhizobium* cultures, by lysing the cells and precipitating the protein with 200 µl of 60% TCA (trichloroacetic acid). These mixtures were vortexed vigorously, then incubated at 4°C for 2 hr and then centrifuged for 10 min. As much of the TCA as possible was removed and the pellets were resuspended in 200 µl of H_2O with vortexing.

The protein content of the resuspended lysed bacterial pellets was determined using the method described by Lowry *et al.* (1951). Stock solutions for protein determination were: solution A (2% Na_2CO_3 in 0.1 M NaOH) made fresh, solution B (2% Na–tartrate in H_2O), solution C (1% $CuSO_4$ in H_2O). Prior to conducting the assay, a fourth solution, D, was made from a mixture of the above three stock solutions in the ratio A:B:C=100:1:1. One millilitre of solution D was added to the 200 µl of protein suspension, mixed by vortexing and incubated at room temperature for 10 min. The reaction was started by the addition of 1 ml 50% Folin's reagent (purchased from E. Merck, Darmstadt, and diluted in H_2O) and immediately mixed with a brief vortex. After a 30 min incubation at room temperature, the absorbance at λ=670 nm was determined. All estimations were performed with three or more repeats and measured against a standard curve of BSA (bovine serum albumin).

2.2.10 Measurement of β-Galactosidase

Rhizobium cultures were grown in their test media to the desired cell density and then assayed for β-galactosidase expression. The promoters under investigation were fused to the *Escherichia coli lacZ* gene carried on the vector pMP220. Addition of tetracycline to the culture media at 0.4 $\mu g.ml^{-1}$ was necessary in order to provide selective pressure for the retention of the plasmid at its maximal copy number. At the time of assaying the β-galactosidase activity, the optical density of the cultures was determined by measuring the absorbance at λ=600 nm. In an eppendorf tube, 400 µl of Z–buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercapto-ethanol, pH 7.5; Miller, 1972) was added to 400 µl of *Rhizobium* culture and the cells were lysed by the addition of 25 µl of SDS (sodium dodecyl sulfate) and 10 µl of $CHCl_3$ followed by vortexing for 30 seconds. The reaction was started by mixing in 160 µl of ONPG solution (4 $mg.ml^{-1}$ *O*–nitrophenol-β-D-galactopyranoside in 0.1 M phosphate buffer, pH 7.0) and terminated after 7.5 min by mixing in 400 µl of 1 M Na_2CO_3. The cell debris was pelleted by centrifugation in an eppendorf centrifuge for 2 min. and the amount of *O*-nitrophenol present was determined by measuring the absorbance of the supernatant at λ=420 nm. All estimations were performed with three or more repeats. The activity of β-galactosidase, normalized against cell density, was calculated using Miller's equation (Miller, 1972).

$$\text{Miller units of } \beta\text{-galactosidase activity} = 1000 \times A_{420} \div (t \times V \times A_{600})$$

where: A_{420} = absorbance at λ=420 nm (determined after the reaction with ONPG),

A_{600} = absorbance at λ=600 nm (measurement of culture density at time of assay),

t = time of ONPG reaction (min.)

V = volume of culture used in the reaction (ml)

2.3 Nucleic Acid Isolation

2.3.1 Large Scale Isolation of Bacterial Genomic DNA (cesium chloride gradient method)

This method produced large amounts clean DNA from both *Rhizobium* and *E. coli* (modified from Chesney *et al.*, 1979). *Rhizobium* was grown on solid TY media to reduce the amount of polysaccharide production. Bacterial cells were collected from confluent lawns on four plates and resuspended in 30 ml of TES (10 mM Tris-HCl, pH 8, 1 mM EDTA, and 100 mM NaCl) and then centrifuged in a SS34 rotor at 6000 rpm for 10 min. at 4°C. Washing the cells in salt removes most of the surface exopolysaccharide and improves cell lysis of *Rhizobium* cells; the salt wash serves no benefit with *E. coli* cells. After centrifugation, the supernatant was discarded and the pellet was resuspended in 4.0 ml of 25% sucrose in 50 mM Tris-HCl (pH 8). The resuspension was transferred to a Corex tube and 0.5 ml of freshly prepared lysozyme solution (10 $mg.ml^{-1}$ in 250 mM Tris-HCl, pH 8) and 1.2 ml 100 mM EDTA (ph 8) was added. The mixture was inverted several times and incubated on ice for 10 min. To this, a 0.6 ml aliquot of freshly prepared SDS solution (10% in H_2O) was added and the mixture was vigorously vortexed for 10 seconds, followed by 250 µl of RNase (2 $mg.ml^{-1}$ ribonuclease A [Sigma] in 100 mM Na-acetate, pH 6.0, and boiled for 15 min.) and a 30 min. incubation at 37°C. A 250 µl volume of proteinase K (Sigma) solution (1 $mg.ml^{-1}$ in H_2O) was added and the samples incubated at 37°C for 60 min. followed by a 16 hr incubation at 55°C. A 0.5 ml aliquot of ethidium bromide (10 $mg.ml^{-1}$) and 7.00 g of CsCl was added to the solution and the CsCl was completely dissolved by gentle inversion mixing of the tubes. The samples, in Corex tubes, were centrifuged at 7000 rpm for 15 min. in a SS34 rotor and then the red transparent lower phase was transferred to a Beckman pollyallomer ultracentrifuge tube (the supernatant should occupy three quarters of the pollyallomer tube). Liquid paraffin was layered above the DNA solution, to a point just below the neck of the pollyallomer tube. Samples were balanced in pairs to within 5 mg using liquid paraffin or when

necessary, with 1.6 g.ml^{-1} CsCl in TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and then the tubes were sealed using a Beckmann "quickseal" apparatus. Samples were centrifuged (F, G or H Beckmann ultracentrifuge) in a Ti50 or Ti80 rotor at 45,000 rpm (with out brakes) for between 48 and 72 hr and at a temperature between 18 and 25°C. At completion of the ultracentrifugation run, the pollyallomer tubes were removed from the rotor with as a little disturbance to the gradient as possible, clamped firmly (not with excessive pressure) in front of an ultraviolet light source (CAUTION: proper protective clothing and eye shields must be worn when exposed to ultraviolet light) and the top of the tube was punctured with a needle. The DNA band was removed from the gradient by gently piercing the pollyallomer tube approximately 1.0 cm below the band with a 19-gauge needle attached to a 2.0 ml syringe; and with the open side of the needle point facing upwards, the DNA band was slowly and uniformly drawn into the syringe. The DNA solution was expelled into a clean Corex tube and the ethidium bromide was extracted from the solution with three equal volume changes of n-butanol. The DNA was dialyzed in a 2 litre volume of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with constant stirring for 24 hr. The buffer was replaced after 30 min., after 3 hr, and after another 3 to 6 hr. The DNA (total volume approximately 2 or 3 ml) was stored in aliquots, at 4°C (for several weeks) and at -20°C (indefinitely). The concentration, endonuclease restrictability and presence of contaminating nucleases could be visualized by agarose electrophoresis of an endonuclease restricted 15 µl aliquot of the sample. The concentration of DNA was some times determined by measuring the absorbance of a 0.01 dilution of the preparation at $\lambda = 260$ nm and comparing this value to that of known standards.

2.3.2 Small Scale Rapid Isolation of Bacterial Genomic DNA

This was a very rapid genomic DNA isolation method, that was done in an eppendorf tube and yielded sufficient DNA for several restriction digests, but was not suitable for cloning experiments. A large lump of cells was scraped off a solid media plate and

placed into an eppendorf tube, such that they occupied a volume of approximately 200 µl. *Rhizobium* cells were resuspended in 1 ml of TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and then centrifuged for 1 min. This was an essential washing step that removed a lot of the exopolysaccharide from the *Rhizobium* cell surface and it was repeated once more. The washed *Rhizobium* cell pellet (or if the sample was *E. coli*, then the 200 µl of collected *E. coli* cells) was resuspended in 500 µl of 25% sucrose in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). A 200 µl volume of lysozyme solution (10 $mg.ml^{-1}$ in 250 mM Tris-HCl, pH 8) was added, followed by a 30 min. incubation at 37°C. A 30 µl volume of 10% SDS was added, followed by 100 µl of proteinase K (10 $mg.ml^{-1}$ in H_2O) and the solution was incubated at 65°C for 16 hr. The sample was extracted five times with an equal volume of a mixture of phenol: chloroform: isoamyl alcohol (in the ratio 25:24:1 and the phenol had been equilibrated against TE, pH 8) by vigorous vortexing for 3 min. and with 10 min. centrifugation spins between each extraction to separate the phases and create a tight interface. After the final extraction, two volumes of ethanol (at $^{-}$20°C) were added to the recovered aqueous phase and the DNA was precipitated at $^{-}$20°C for 2 to 16 hr. The precipitated DNA was pelleted by a 6 min. centrifugation, dried under vacuum for 15 min. and resuspended in 100 µl of TE buffer. The concentration, endonuclease restrictability and presence of contaminating nucleases could be visualized by agarose electrophoresis of an endonuclease restricted 15 µl aliquot of the sample; and the remainder of the DNA was stored at 4°C.

2.3.3 Large Scale Isolation of Supercoiled Plasmid DNA (cesium chloride gradient method)

This method (modified from Clewell and Helinski, 1969) was used for obtaining several milligrams of ultra-pure supercoiled plasmid DNA from *E. coli*; that was suitable for any enzyme reaction, including double stranded sequencing and S1 nuclease promoter mapping. Confluent lawns of the plasmid bearing strain were set up by spreading 2 ml

of fresh liquid culture over a solid LB plate and incubating the plate at 37°C for 16 hr. The cells from two plates were scraped off the agar surface using a glass rod that had been bent into a loop, and placed into a SS34 tube containing 8.0 ml of 25% sucrose in 50 mM Tris-HCl (pH 8). The cells were resuspended by vigorous vortexing, 4.5 ml of 250 mM EDTA (pH 8) and 0.5 ml of freshly prepared lysozyme solution (40 $mg.ml^{-1}$ in TE) were added, then the solution was mixed and incubated on ice for 10 min. A 4.5 ml volume of 2% Triton X 100 detergent was added, followed by a quick inversion of the tube for mixing. This solution was left for 30 min. on ice and the solution became very viscous, indicating that cell lysis had occurred. Pairs of tubes were balanced by addition of 25% sucrose in 50 mM Tris-HCl (pH 8), then centrifuged at 18,000 rpm for 60 min. in a SS34 rotor at 4°C. The supernatant was transferred to a clean SS34 tube and 6 ml of 5 M NaCl and 7.5 ml of 40% PEG-6000 (in 50 mM Tris-HCl, pH 8, 20 mM EDTA) were added to the solution; then the sample was mixed by inversion and incubated on ice for 16 hr. Paired tubes were balanced by the addition of 25% sucrose in 50 mM Tris-HCl (pH 8) and then centrifuged at 7000 rpm for 10 min. in a SS34 rotor at 4°C. The supernatant was discarded and as much of the PEG as possible was removed, while being careful not to dislodge the DNA pellet. The precipitated DNA was resuspended in 6.4 ml of TES buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl). The resuspended DNA was added to a Corex tube already containing 7.00 g of CsCl and to this, 0.9 ml of ethidium bromide (10 $mg.ml^{-1}$) was added. Mixing the contents of the tube by inversion, eventually dissolved the CsCl and then the solution was incubated on ice for 30 min. The samples were then centrifuged at 10,000 rpm in a SS34 rotor at 4°C for 30 min. The supernatant was transferred to a Beckman pollyallomer ultracentrifuge tube and the remaining quarter of the pollyallomer tube was filled with liquid paraffin to a point just below the neck of the tube. Samples were balanced in pairs to within 5 mg using liquid paraffin or when necessary, with 1.6 $g.ml^{-1}$ CsCl in TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and then the tubes were sealed using a Beckmann "quickseal" apparatus. Samples were

centrifuged (F, G or H Beckmann ultracentrifuge) in a Ti50 or Ti80 rotor at 45,000 rpm (with out brakes) for between 48 and 72 hr and at a temperature between 18 and 25°C. At completion of the ultracentrifugation run, the pollyallomer tubes were removed from the rotor with as a little disturbance to the gradient as possible, clamped firmly (not with excessive pressure) in front of an ultraviolet light source (CAUTION: proper protective clothing and eye shields must be worn when exposed to ultraviolet light) and the top of the tube was punctured with a needle. Two bands of DNA were visible; the lower band is supercoiled plasmid DNA and the upper band is largely chromosomal DNA. The lower DNA band was removed from the gradient by gently piercing the pollyallomer tube approximately 1.0 cm below the lower band with a 19-gauge needle attached to a 2.0 ml syringe; and with the open side of the needle point facing upwards, the plasmid DNA band was slowly and uniformly drawn into the syringe. The plasmid DNA solution was expelled into a clean Corex tube and the ethidium bromide was extracted from the solution with three equal volume changes of n-butanol. The plasmid DNA was dialyzed in a 2 litre volume of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with constant stirring for 24 hr. The buffer was replaced after 30 min., after 3 hr, and after another 3 to 6 hr. The plasmid DNA (total volume approximately 2 or 3 ml) was stored at -20°C. The concentration, endonuclease restrictability and presence of contaminating nucleases or chromosomal DNA could be visualized by agarose electrophoresis of an endonuclease restricted 10 µl aliquot of the sample.

2.3.4 Small Scale Rapid Isolation of Plasmid DNA from *E. coli* (alkaline lysis method)

This method was very useful for the rapid screening recombinant plasmids, since the entire procedure is performed in eppendorf tubes at the bench; and in only two hours, 12 samples can be taken from live cells to purified plasmid DNA (modified from Birnboim and Doly, 1979). A single recombinant clone was inoculated into 5 ml of LB liquid media and grown for 16 hr at 37°C with shaking. A 1.5 ml eppendorf tube was filled

with dense culture and centrifuged for 2 min. The supernatant was discarded, the cell pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl, pH 8, 10 mM EDTA) and then incubated at room temperature for 5 min. A 200 µl volume of freshly prepared solution II (200 mM NaOH, 1% SDS) was added, followed by a 5 min. incubation on ice, then 150 µl of solution III (potassium acetate made up as 3 M CH_3COOK in 5 M CH_3COOH) was added and followed by a 10 min. incubation on ice. The sample was centrifuged for 3 min. and the supernatant was transferred to a clean eppendorf tube, where it was extracted against an equal volume of a mixture containing phenol: chloroform: isoamyl alcohol (in the ratio 25:24:1 and the phenol had been equilibrated against TE, pH 8). After a 5 min. centrifugation, the top 80% of the aqueous phase (with care not to remove any of the organic phase) was transferred to a clean eppendorf tube. The DNA was precipitated by adding two volumes of 100% ethanol (at $^{-}20°C$), mixing, incubating at room temperature for 2 min. and finally centrifuging for 3 min. All of the supernatant was removed with a drawn out pasteur pipette and discarded. The DNA pellet was dried under vacuum for 15 min. and then resuspended in 200 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) with vortexing. Typically for pUC based plasmids, only 1 µl was required for agarose gel electrophoresis.

2.3.5 Small Scale Isolation of Plasmid DNA from *Rhizobium*

This method was used to isolate plasmid DNA from *Rhizobium* transconjugants for the purposes of restriction analysis or transformation into *E. coli*. The method is only slightly different from that described in section 2.3.4. The *Rhizobium* clone was inoculated into 5 ml of liquid TY media, supplemented with plasmid selecting antibiotics and incubated at 30°C with shaking for 24 hr. A 3 ml volume of the culture was pelleted into a single 1.5 ml eppendorf tube with two consecutive 2 min. centrifugations of a full tube. The supernatant was discarded after both spins and the combined cell pellet was resuspended in 200 µl of solution I, incubated at room temperature for 5 min.; 400 µl

of solution II was added, mixed and followed by a 15 min. incubation on ice; a 300 µl volume of solution III was added, incubated on ice for 20 min. and then centrifuged for 4 min. The supernatant was transferred to a clean tube and centrifuged again for 4 min. The supernatant was transferred to another clean eppendorf tube and extracted against an equal volume of a mixture containing phenol: chloroform: isoamyl alcohol (in the ratio 25:24:1 and the phenol had been equilibrated against TE, pH 8), by vortexing vigorously for 3 min., followed by a 10 min. centrifugation and the aqueous phase transferred to a clean tube. The extraction was repeated another 3 times. After the final extraction, the DNA was precipitated by addition and mixing in, of an equal volume of 100% isopropanol, followed by a 10 min. incubation at room temperature and a 5 min. centrifugation. The supernatant was discarded and the pellet was resuspended in 500 µl of TE, without drying the DNA. This DNA solution was extracted twice in the usual way with the phenol-chloroform solution, being especially careful not to remove any of the organic phase with the recovery of the aqueous phase after the final extraction. The DNA solution was made up to 300 mM Na-acetate from a stock of 3 M Na-acetate, thoroughly mixed, and two volumes of ethanol (⁻20°C) were added with mixing. The tubes were then incubated in dry ice for 30 min. and then centrifuged for 10 min. The supernatant was entirely removed using a drawn out pasteur pipette; the pelleted DNA was vacuum dried for 15 min. and then resuspended in 60 µl of TE buffer. The DNA yield was sufficient for approximately 4 restriction digests the DNA was stored indefinitely at ⁻20°C.

2.3.6 Extraction of RNA from *Rhizobium*

In an effort to avoid RNase contamination: all solutions were prepared with sterile, deionized, distilled, Milli Q water; they were stored in new washed and sterilized glass-ware or in sterilized disposable plastic-ware; they were filter sterilized and autoclaved each time before use; gloves were worn during the entire extraction procedure and all manipulations were done on ice-salt baths (unless otherwise indicated). A mid log phase

culture of *Rhizobium* (OD_{600} between 0.5 and 1), grown in 500 ml of BMM liquid media at 30°C, was harvested in Sorvall SS34 centrifuge tubes at 10,000 rpm, at 4°C, for 5 min. The pellets were resuspended and washed in TES (100mM NaCl, 1mM EDTA, 10mM Tris-HCl, pH7.4) to aid in the removal of exopolysaccharide from the cell surface and then pelleted again at 10,000 rpm, at 4°C, for 5 min. All pellets were resuspended in TES to a combined total volume of 15 ml, then transferred to eppendorf tubes and centrifuged for 1 min. After discarding the supernatant, the bacterial pellet of each eppendorf tube was resuspended in 500 µl of extraction buffer (10 mM Tris-HCl, pH 7.5, 5 mM EDTA, 5% sucrose, 300 mM CH_3COONa, 1% SDS, 1% 2-β-mercaptoethanol). A 300 µl volume of phenol (equilibrated against TE, pH 8) at 90°C was added, mixed, followed by 300 µl of $CHCl_3$-isoamyl alcohol (24:1). The tubes were vortexed and centrifuged for 10 min. The aqueous phase was recovered and the nucleic acid precipitated; by addition of 2.5 volumes of 100% ethanol, followed by a 30 min. incubation on dry-ice, and a 6 min. centrifugation at 4°C. The supernatant was removed and the pellet was resuspended in 50 µl of TE. An equal volume of 8 M LiCl was added and RNA was precipitated for 16 hr at -20°C. Insoluble RNA was centrifuged for 15 min. at 4°C and resuspended in 50µl of TE with vortexing. The LiCl precipitation was repeated to eliminate contaminating DNA. The final RNA pellet was resuspended in 100 µl of TE and stored as two 50 µl aliquots at -20°C. To determine the yield and condition of the RNA, a 5% aliquot was visualized on a MOPS-agarose gel.

2.3.7 Isolation of Single Stranded Phagemid DNA for Sequencing

Single stranded DNA was isolated from Bluescript recombinants using the helper-phage VCSM13 by the following protocol. A single colony of the relevant bluescript recombinant clone and 1µl of VCSM13 helper-phage ($1x10^{13}$ $pfu.ml^{-1}$) was inoculated into 2 ml of liquid LB supplemented with 50 $\mu g.ml^{-1}$ ampicillin and shaken at 37°C. After two hours kanamycin was added to the culture to make a final concentration of 70 $\mu g.ml^{-1}$ and the culture was shaken at 37°C for 16 hours. The phage particles were

separated from the bacterial cells by centrifuging the culture for 5 min. at 4°C in a 1.5ml eppendorf tube. The supernatant was transferred to another eppendorf tube and centrifuged a second time for 5 min. at 4°C. The supernatant was again transferred to another tube containing 300 µl of a solution of 25% PEG and 2.5 M NaCl; incubated on ice for 30 min., and then centrifuged at 4°C for 5 min. The phage pellet was resuspended in 100 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). To the resuspension, 50 µl of phenol (equilibrated against TE, pH8) was added and the mixture vortexed for 1 min., followed by a 10 min. centrifugation at room temperature. An 80µl volume of the aqueous phase was recovered and the ssDNA was precipitated by addition of 4 µl of 3 M Na-acetate and 180 µl of 100% ethanol, mixed, incubated at room temperature for 2 min. and then centrifuged for 6 min. The supernatant was discarded and the DNA pellet was washed with 70% ethanol (⁻20°C) by slowly filling the eppendorf tube with the 70% ethanol, centrifuging for 3 min. and removing the supernatant with a drawn-out pasteur pipette. The ssDNA pellet was dried under vacuum for 12 min., resuspended in 50 µl TE and stored at ⁻20°C. The concentration and quality of the preparation was examined by agarose gel electrophoresis of a 3 µl aliquot.

2.3.8 Isolation of Single Stranded M13 Phage DNA for Sequencing

Single stranded DNA from M13 recombinant phages was isolated by the method of Heidecker *et al.* (1980). The appropriate recombinant plaque and 20 µl of fresh *E. coli* strain JM107 culture were inoculated into 2 ml of liquid LB media and incubated at 37°C with shaking for 5 hr. A 1.5 ml volume of the culture was centrifuged in an Eppendorf bench centrifuge for 1 min. and 1.2 ml of the supernatant was transferred to a clean eppendorf tube and then centrifuged for 10 min. A 1.1 ml volume of the supernatant was transferred to an eppendorf tube containing 300 µl of a solution (20% PEG-6000, 2.5 M NaCl), thoroughly mixed and incubated on ice for 20 min. The phage particles were pelleted with a 3 min. centrifugation, all of the PEG was removed from the pellet, which was then resuspended in 300 µl of TES (100mM NaCl, 1mM EDTA, 10mM

Tris-HCl, pH7.5) with vortexing. The resuspended phage solution was extracted twice with 250 µl of phenol-chloroform, by vigorous vortexing and separating the phases with a 3 min. centrifugation. With the recovered aqueous phase in a new eppendorf tube, 20 µl of 3 M Na-acetate was added and mixed, followed by the addition and mixing of 2 volumes of 100% ethanol (⁻20°C). The ssDNA was precipitated for 16 hr at ⁻20°C and centrifuged for 10 min. The pellet was dried under vacuum for 15 min., then resuspended in 30 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and stored at ⁻20°C. The concentration and quality of the preparation was examined by agarose gel electrophoresis of a 3 µl aliquot.

2.4 Enzymatic Reactions

2.4.1 Restriction Endonuclease Digestion

Restriction endonucleases were usually purchased from Boehringer Mannheim GmbH W. Germany or from New England Biolabs, UK. The reaction conditions were always those that were recommended by the manufacturers, and most of the conditions are summarized as follows: TA buffer (33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM DTT); Low Salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT); Medium Salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT); High Salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT). These buffers were prepared in advance as 5 X stocks and stored at ⁻20°C for many weeks. Usually 2 µg of DNA was digested in a final volume of 20 µl with sufficient endonuclease to fully digest the DNA in 1 hr at 37°C (unless otherwise stated). When double or multiple digests were carried out and the NaCl requirements of each endonuclease were not compatible, the DNA was restricted first with the endonuclease requiring the lesser [NaCl], then sufficient NaCl was added to enable optimum activity for the second enzyme and the endonuclease reaction continued.

2.4.2 Dephosphorylation of Vector DNA

Calf intestinal alkaline phosphatase (CIP), 1 unit.μl^{-1} was purchased from Boehringer Mannheim GmbH W. Germany. CIP catalyses the removal of 5'-phosphate residues from DNA. The plasmid DNA to be phosphatased was first completely restricted with the appropriate endonuclease and then the volume of the reaction mixture was increased to 200 µl, including the addition of 20 µl of a 10 X stock of phosphatase buffer (10 X buffer is; 500 mM Tris-HCl, pH 9, 1 mM EDTA, 10 mM $MgCl_2$) and CIP (2 units.μg^{-1} of DNA). The reaction mixture was incubated at 37°C for 20 min. and then terminated by increasing the volume to 500 µl and extracting with: an equal volume of phenol-chloroform, an equal volume of chloroform and then two extractions with ether. Each extraction involved gently mixing the solution by inversion, then centrifuging for 5 min. and then removing the aqueous phase to a clean eppendorf tube. The DNA was precipitated by **standard ethanol DNA precipitation method** (in an eppendorf tube), which meant that Na-acetate was added to a total concentration of 300 mM (from a stock of 3 M CH_3COONa), mixed, then 2 volumes of ethanol ($^-$20°C) was added, mixed, the solution incubated at $^-$20°C for 2 hr, centrifuged for 6 min., the supernatant removed using a drawn out (by hand, over a bunsen burner, in order to make a smaller bore diameter) glass pasteur pipette, and the DNA pellet was dried under vacuum for 12 min. The pellet was resuspended in 20 µl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA).

2.4.3 Ligation of DNA Molecules

T4 DNA ligase catalyses the formation of a phosphodiester bond between adjacent 3'-OH and 5'-phosphate termini in DNA. Prior to ligating DNA, it is necessary to denature the restriction endonucleases that are present; for some enzymes this can be achieved by heating to 85°C for 8 min. (see manufacturers' details) and others require phenol-chloroform extraction followed by ethanol precipitation. The buffer conditions used for ligation of compatible cohesive ends was 66 mM Tris-HCl (pH 7.6), 6.5 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP; for blunt end ligations, the [ATP] was reduced to

0.1 mM. The ATP has a limited active life span and was stored separately as a 20 mM stock solution at -20°C, while the remaining components of the buffer were stored as a 5 X stock at -20°C. Ligations were not affected by the restriction endonuclease buffers that were often present in the DNA samples, but they were affected by contaminating traces of agarose or phenol. The typical reaction volume for the ligation of two fragments was 30 µl; it was 200 µl for recircularizing a single linear fragment. A ligation reaction involved mixing the DNA samples together with the ligation buffer (from a 5 X stock, including fresh ATP), T4 DNA ligase (0.5 units.μg^{-1} DNA) and the reaction mixture was incubated at 16°C for 16 hr.

2.4.4 Kinasing of DNA 5' Termini (End-Labelling)

T4 polynucleotide kinase (PNK), 8 units.μl^{-1}, was purchased from Boehringer Mannheim GmbH W. Germany. PNK catalyses the transfer of the γ-phosphate of ATP to a 5'-OH terminus in DNA and was thus used to radioactively end-label the 5' ends of DNA fragments that had been previously dephosphorylated (2.4.2), or to end-label single stranded oligodeoxynucleotides that do not have a 5'-phosphate. The radioactively labelled nucleotide substrate was adenosine 5'-[γ-^{32}P]-triphosphate (>5000 Ci.$mmol^{-1}$) purchased from Amersham, Buckinghamshire, England. For both end-labelling reactions, the PNK reaction conditions were 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM DTT (as recommended by the manufacturers) and this kinase buffer was stored as a 5 X stock at -20°C. Between 1 and 10 µg of DNA was kinased by mixing the DNA with the kinase buffer, 1 µl PNK, 2 µl [γ-^{32}P]-ATP, in a total volume of 30 µl, and the reaction mixture was incubated at 37°C for 1 hr. End-labelled DNA fragments used for S1 promoter mapping were restricted with another endonuclease and electrophoresed through agarose (see 2.4.10).

End-labelled oligodeoxynucleotides were separated from the unused [γ-^{32}P]-ATP by anion exchange chromatography on a DEAE Sephadex A-25 (Bio-Rad) column. A 2 cm

column was set up in a pasteur pipette and equilibrated with buffer A (TE + 50 mM NaCl). The sample was made up to a volume of 200 µl with TE, loaded onto the column and washed in with 3 ml of buffer A. The unused [γ-^{32}P]-ATP was eluted off with 20 ml of buffer B (TE + 200 mM NaCl). Finally the probe was eluted off with buffer C (TE + 1 M NaCl) and collected in 100 µl fractions.

2.4.5 Radioactive Labeling of DNA Fragments (random primer method)

Radioactively labelled hybridization probes were prepared by randomly primed synthesis of DNA using *E. coli* DNA polymerase I (Klenow fragment; BRESA, Australia) by a method similar to that described by Whitfeld *et al.* (1982). Random primers of 8-12 nucleotides in length were prepared by treating herring sperm DNA with DNase I and fractionation on a DEAE-Sephadex G-50 column (Taylor *et al.*, 1976). The radioactive probe was prepared by restricting the DNA fragment or plasmid (approximately 1 to 5 µg of DNA) with *Hae*III in 2 X TA buffer (1 X; 33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM DTT) in a total volume 15 µl for 10 min. at 37°C. A 1 µl volume of random primers (approximately 20 µg) was added to the reaction and the solution was boiled at 100°C for 2 min. The reaction tube was transferred from 100°C immediately to an ice bath for 2 min. The reaction was made up to a 30 µl volume including the addition of three cold deoxynucleotide triphosphates (1 µl of 20 mM dATP, 1 µl of 20 mM dGTP and 1 µl of 20 mM dTTP), 0.5 µl of DNA polymerase I (5 units.µl^{-1}) and 2 µl of 5'-[α-^{32}P]-dCTP (3000 Ci.mmol^{-1}; Amersham, England). The reaction was incubated at 37°C for 60 min. and then terminated by the addition of 10 µl of tracking dye (0.25% bromophenol blue, 35% sucrose, 40% glycerol, 10 mM Tris-HCl, pH 8, 1 mM EDTA). The labelled DNA was separated from the unincorporated ^{32}P-nucleotides by passing the reaction mixture through a Sephadex G-50 column (packed bed volume of 5 ml). The column was equilibrated with TES (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 100 mM NaCl) and

had a flow rate of 1 ml.min^{-1}. Fractions (300 µl) were collected in eppendorf tubes, the labelled DNA was eluted just after the void volume and was monitored using a Geiger counter. Immediately prior to addition of the probe to hybridization mixtures, the probe was denatured by boiling for 2 min. followed by cooling on ice for 2 min.

2.4.6 End-Filling of DNA 3'-Termini (^{32}P-labeling)

This method was used to radioactively label the 3' ends of DNA fragments, but otherwise leaving the double stranded DNA fragment intact. The DNA was restricted with an appropriate endonuclease (usually in a 20 µl volume of TA buffer) that leaves a single stranded 5' over-hang of a few nucleotides. A 1 µl volume of DNA polymerase I (5 units.µl^{-1}) was added and incubated for 20 min at 37°C to allow some 3'→5' exonuclease activity. The reaction volume was made up to 40 µl in TA buffer, which also included the addition of 2 µl of either 5'-[α-^{32}P]-dCTP or 5'-[α-^{32}P]-dATP (whichever was complimentary to the 5' single stranded DNA overhang created by the restriction endonuclease), and 2 µl of each of the other three cold nucleotides (stock concentrations were 20 mM). The reaction was incubated at 37°C for 30 min. and then 2 µl of cold 20 mM nucleotide (identical to the ^{32}P-nucleotide in the previous step) was added, followed by a further incubation at 37°C for 10 min. (chase step). Generally, 3' end-labeling of fragments was used to facilitate the detection of the fragment after electrophoresis and thus, it was not necessary to remove the unincorporated ^{32}P-nucleotides from the reaction. However when necessary, the end-labelled DNA can be separated from unincorporated ^{32}P-nucleotides using the Sephadex G-50 column described in 2.4.5.

2.4.7 End-Filling of DNA Termini to Generate Blunt Ends

Recessed 3' ends generated by some restriction endonucleases, were filled in to produce blunt ends that could then be ligated with other blunt ends. This facilitated the ligation of DNA molecules at sites that produce incompatible single stranded DNA over-hangs. The

method is similar to that described in 2.4.6; and involves mixing the restricted DNA molecules (usually a post-digestion volume of 20 µl), with 8 µl of solution containing all four nucleotides (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 5 mM dTTP), 1 µl of DNA polymerase I (5 units.μl^{-1}) and making up to final volume of 40 µl with TA buffer (33 mM Tris-acetate, pH 7.9, 10 mM Mg-acetate, 66 mM K-acetate, 0.5 mM DTT). The reaction mixture was incubated at 37°C for 30 min. The reaction volume was increased to 500 µl and was terminated along with the removal of restriction endonucleases by extracting with: an equal volume of phenol-chloroform, an equal volume of chloroform and then two extractions with ether. Each extraction involved gently mixing the solution by inversion, then centrifuging for 5 min. and then removing the aqueous phase to a clean eppendorf tube. The DNA was precipitated by standard ethanol DNA precipitation method (see 2.4.2) and was resuspended in 20 µl of TE.

2.4.8 DNA Sequencing from a ssDNA Template

(i) Bluescript sequencing kit (Stratagene)

This was the most effective method, because of the convenience of the Bluescript SK^+ and SK^- vector system for the generation and manipulation of sequencing clones. The DNA sequencing protocol provided with this kit (United States Biochemical Corporation) is a modification of the dideoxy chain termination technique (Sanger *et al*, 1977), which uses a modified T7 DNA polymerase (described by Tabor and Richardson, 1987). The kit was used according to the manufacturers instructions except that a standard chase step of 1µl of chase mix (1mM of each dNTP) was included. Template DNA was isolated by the method described in section 2.3.7 and 3 µl was used per sequencing reaction.

(ii) Sequencing with M13 Bacteriophage

These sequencing reactions were carried out using the dideoxy chain termination technique of Sanger *et al.* (1977), using the Klenow fragment of *E. coli* DNA polymerase I and recombinant M13mp18 and M13mp19 recombinants. The template

ssDNA was isolated as described in section 2.3.8 and the primer is described in section 2.1.7. The primer was annealed to the template by mixing 8 µl of template DNA, 1 µl of primer, 2 µl of 10 X RT buffer (10 X is 600 mM Tris-HCl, pH 7.5, 80 mM $MgCl_2$, 100 mM DTT), incubating the mixture at 70°C for 5 min. and then allowing the mixture to cool slowly to room temperature over 30 min. To this annealed DNA was added 1 µl of DNA polymerase I (5 units.µl^{-1}), 1 µl of 5'-[α-^{32}P]-dATP (3000 Ci.mmol^{-1}, Amersham) and the contents were mixed. This mixture was divided into four 2.5 µl aliquots, each of which were transferred to separate eppendorf tubes. The four receiving eppendorf tubes already contained 1 µl of only one of the following:

tube 1 - G mix (24 µM dGTP, 5 µM dATP, 50 µM dTTP, 50 µM dCTP, 250 µM ddGTP);
tube 2 - A mix (50 µM dGTP, 24 µM dATP, 50 µM dTTP, 50 µM dCTP, 21 µM ddATP);
tube 3 - T mix (50 µM dGTP, 5 µM dATP, 10 µM dTTP, 50 µM dCTP, 250 µM ddTTP);
tube 4 - C mix (50 µM dGTP, 5 µM dATP, 50 µM dTTP, 45 µM dCTP, 250 µM ddCTP).

(For each of the reaction mixes, it was necessary to adjust the deoxy: dideoxy nucleotide ratios, in order to optimize the chain termination characteristics.)

The reaction tubes were incubated at 37°C for 15 min. and then 1 µl of chase mix (1 mM of each dNTP) was added and the reaction continued at 37°C for a further 10 min. The reaction was terminated by the addition of 4 µl of formamide loading dye (90% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 1 mM EDTA, 10 mM Tris-HCl, pH 8). The DNA was denatured by heating at 95°C for 2 min. and chilling on ice, prior to loading a 2.0 µl sample onto a DNA sequencing gel (7 M urea, 5% acrylamide, see 2.5.3).

2.4.9 DNA Sequencing from a Double Stranded Plasmid Template

Plasmid DNA that was prepared by the cesium chloride gradient method (2.3.3) and was free of RNA, could be used as a template for dideoxy chain termination sequencing. The

only difference between this method and the one described in section 2.4.8 is the annealing of the primer to the template, whereas the sequencing reactions are identical. A 10 µl volume of denaturation solution (0.4 M NaOH, 4 mM EDTA) was added to 10 µl of plasmid DNA (0.2 $\mu g.\mu l^{-1}$) and this was incubated at room temperature for 5 min. To this was added 15 µl of 1 M Na-acetate (pH 4.5), 15 µl of H_2O, 125 µl of 100% ethanol ($^{-}$20°C), followed by a 6 min. centrifugation to precipitate the DNA. The DNA was washed in 70% ethanol ($^{-}$20°C), centrifuged again for 3 min. and the pellet dried under vacuum for 10 min. The template DNA was resuspended in 8 µl of H_2O, 2 µl of 10 X RT buffer, 1 µl of primer oligonucleotide and the mixture incubated at 37°C for 15 min. The subsequent polymerase reactions are identical to those described in section 2.4.8.

2.4.10 Mapping Transcription Initiation Sites Using S1 Nuclease.

The S1 nuclease (300 $units.\mu l^{-1}$) was purchased from Boehringer Mannheim and the method was a modification of that recently described by Calzone *et al.* (1987). The DNA probe for the *exoY* transcript was end-labelled at the *Sma*I site at nucleotide position 1700 (see Fig. 4.10), within the *exoY* coding region (see Fig. 4.8). The probe used to identify the transcribed region without any open reading frames (ORFs) was end-labelled at the *Eco*RI site at nucleotide position 789 (see Figs. 4.8 and 4.10). Restriction enzyme sites were 5'-end labelled with [γ-^{32}P]ATP using polynucleotide kinase (2.4.4) following dephosphorylation by calf intestine alkaline phosphatase (2.4.2). The double stranded probe fragment, now end-labelled at its two 5' ends, was restricted at an appropriate restriction site to free the probe fragment labelled only at the 5' end of the antisense strand. This fragment was recovered from an agarose gel slice by centrifugation in a TLS55 Beckman rotor at 55,000 rpm for 2 hr at room temperature (see 2.5.4.ii).

Rhizobium RNA was isolated from strain ANU240 (2.3.6) and a 50 µl aliquot (2 $\mu g.\mu l^{-1}$) was pooled with the appropriate kinased probe fragment (1 µg) and

precipitated by the standard ethanol DNA precipitation method (2.4.2). The pellet was resuspended in 10 µl of annealing buffer (80% formamide, 80mM sodium piperazine-N,N'-bis-2-ethanesulfonic acid (pH6.8), 400mM NaCl, 10mM Na_2-EDTA) in an eppendorf tube and the solution was covered with 10 µl of fresh paraffin oil to prevent evaporation. The mixture was incubated at 85°C for 15 min. to denature the nucleic acid and then the water-bath was slowly cooled to 58°C for hybridization* and incubated at that temperature for 18 hr. The annealing reaction was terminated by placing the tubes on ice for 2 min; followed by the addition of 200 µl of ice-cold S1 nuclease solution (30 mM Na-acetate, pH 4.5, 280 mM NaCl, 1 mM $ZnSO_4$, 20 $\mu g.ml^{-1}$ freshly denatured calf thymus DNA, 20 $\mu g.ml^{-1}$ native calf thymus DNA, 1.5 $units.\mu l^{-1}$ S1 nuclease). The S1 nuclease solution was squirted in hard to displace the paraffin oil, mixed and then incubated at 37°C for 30 min. The S1 nuclease digestion reaction was terminated by transferring the digestion solution (avoiding the paraffin) to 20 µl of stop solution (500 mM Tris-HCl, pH9, 100 mM EDTA and 50 $\mu g.ml^{-1}$ tRNA). A 550 µl volume of 100% ethanol was added to the terminated reaction mixture and the nucleic acid was pelleted by centrifuging for 6 min. The pellet was washed in 70% ethanol and centrifuged for 3 min. The supernatant was discarded, but the pellet was not dried; it was immediately resuspended (required vigorous vortexing) in 10 µl of H_2O and 20 µl of formamide loading dye (0.1% bromophenol blue, 0.1% xylene cyanol in recrystallized formamide). The H_2O was dried off using a spin-vac (low velocity centrifuge with vacuum applied); and the sample was heated to 90°C for 4 min. just prior to loading on an 8M urea-3% (20:1, acrylamide: bis) denaturing polyacrylamide gel (prepared as described in 2.5.3). Size standards were 3'-^{32}P-labelled, end-filled *Cla*I restricted lambda DNA (2.4.6); these were electrophoresed along side the S1 promoter mapping samples to permit the length of the protected fragment to be calculated. The samples were loaded into wells at the top of the gel as opposed to lanes created with a "shark tooth" comb (see 2.5.3) and electrophoresed at 2000 V (60 mA, 100 W).

* The optimal hybridization temperature was determined using formulae and rational presented by Casey and Davidson (1977) and Amersham Corp. (1985). The optimal hybridization temperature for a DNA-DNA hybrid was calculated using the formula:-

$$Tm = 81.5°C+16.6xlogM+0.41x(\%G+\%C)-500\div n -0.61x(\%formamide)$$

M = ionic strength of the hybridization solution (0.4 M)

n = the length of the annealed region

RNA:DNA hybrids are more stable than DNA:DNA hybrids and an extra 9°C was added onto the calculated Tm to allow for this.

2.5 Gel Electrophoresis and Manipulations

2.5.1 Agarose Gel Electrophoresis of DNA

DNA was electrophoresed on horizontal agarose slab gels in Tris Borate Electrophoresis (TBE) buffer (50 mM Tris, 40 mM H_3BO_3, 1 mM EDTA, pH 8.3). The agarose used was Seakem le Agarose (FMC corporation, Rockland, USA). The density of the agarose gel varied from 0.7% to 2.0% (w/v) depending upon the sizes of the DNA fragments to be separated; 2.0% gels provided good resolution for fragment sizes between 100 and 500 bp, 0.7% gels provided good resolution for sizes between 1 and 20 kb. The gels were prepared by dissolving the agarose in TBE buffer by bringing the solution to a boil in a microwave oven, and then pouring the molten agarose into a slab mould. Horizontal agarose gel electrophoresis was carried out essentially as described by Maniatis *et al.* (1982, p 150-162). Two gel slab dimensions were used: maxi gel; 190 x 130 x 7 mm, and the mini gel; 85 x 53 x 7 mm. Typically, 1.5 µg of DNA in a volume of 20 µl was loaded into a maxi gel well, and 0.5 µg of DNA in a volume of 7 µl was loaded into a mini gel well. Prior to loading a sample, a one tenth volume of tracking dye (0.25% bromophenol blue, 35% sucrose, 40% glycerol, 10 mM Tris-HCl, pH 8, 1 mM EDTA) was added. Immediately after loading all the samples for a particular gel, the electric current was applied: maxi gels; 25 V, 16 mA (usually 16 hr), and mini gels; 80 V, 30 mA (approximately 70 min.). After electrophoresis, the agarose gels were immersed

in a solution of ethidium bromide (5 $\mu g.ml^{-1}$) for 15 min; rinsed in distilled water and finally photographed. The fluorescent bands were photographed over a 302 nm wave length ultraviolet transilluminator, using Polaroid type 665 or 667 film with a yellow filter. Size standards such as *Hin*dIII restricted bacteriophage lambda DNA were run in parallel to the experimental samples. The rate of migration of a DNA fragment during gel electrophoresis is inversely proportional to the logarithm of its length.

2.5.2 Agarose Gel Electrophoresis of RNA

Every effort was made to keep all solutions free of contaminating RNases. The quality and yields after RNA extraction from bacteria (2.3.6) were examined by electrophoresis of an aliquot of the RNA through 1% agarose in MOPS buffer (20 mM morpholine-propane-sulfonic acid, 5 mM Na-acetate, pH 7, 1 mM EDTA, 0.05% ethidium bromide). Usually 2 µl of RNA (2 $\mu g.\mu l^{-1}$) was added to 5 µl loading buffer (50% formamide, 18% formaldehyde, 5% glycerol, 0.1% bromophenol blue, in 1 X MOPS buffer), the sample loaded onto a mini gel (2.5.1) and electrophoresed at 40 V (15 mA) for 2.5 hr and with periodic (every 45 min.) mixing of the buffer. After electrophoresis, the gel was photographed immediately since the ethidium bromide was incorporated into the MOPS electrophoresis buffer. All other details about RNA electrophoresis through agarose were identical to DNA electrophoresis through agarose (2.5.1).

2.5.3 Polyacrylamide Gel Electrophoresis of DNA

The following is a description of the methods used for electrophoresis of DNA sequencing reactions; any variations relevant to the electrophoresis of S1 nuclease protected DNA fragments are given in that section (2.4.10). The products of DNA sequencing reactions (2.4.8 and 2.4.9) were resolved in thin, denaturing polyacrylamide gels (5% acrylamide:bis-acrylamide [20:1], 7 M urea). To prepare 1000 ml of the gel solution, 420 g of urea was dissolved in 200 ml of 10 X TBE buffer (10 X is: 500 mM Tris, 400 mM H_3BO_3, 10 mM EDTA, pH 8.3) and 300 ml H_2O by warming

in a microwave oven. A 125 ml volume of 40% acrylamide:bis-acrylamide (20:1) was added, the solution filtered through Whatman 3MM paper, the volume made up to 1000 ml with distilled H_2O and the solution was stored for up to 2 weeks at 4°C. For each gel, 150 mg of ammonium persulfate was dissolved in 150 ml of the gel solution and immediately before pouring the gel, 67 µl of TEMED (N,N,N',N'-tetramethyl-ethylenediamine) was added. The mixture was poured into a mould created by placing two 60 x 35 cm glass plates (6 mm thick) together, but separated with a thin wedge shaped spacer (0.3 mm at the top and 0.5 mm at the base) and the whole system was sealed along the sides and bottom edge with tape, to hold the liquid unpolymerized gel. The gel was allowed to polymerize for several hours and was then set up as a vertical electrophoresis system, buffered with 2 X TBE (no urea) and an aluminium plate (1 mm thick) was installed against the front glass plate to aid in heat dissipation. Sample lanes were separated by placing a tightly fitting "shark tooth" comb at the cathode end of the gel. The loading compartments were flushed with buffer in order to remove the exuding urea, and 2.0 µl of a single sequencing reaction (2.4.8 or 2.4.9) was loaded into each compartment. The reactions were electrophoresed at 1800 V (55 mA, 90 W) for approximately 4 hr to resolve the first 250 nucleotides and 8 hr for the next 200 nucleotides. After electrophoresis, the glass plates were separated and the gel transferred to Whatman 3MM paper, covered with plastic wrap, and dried at 80°C on a vacuum gel drier for 60 min. prior to autoradiography at room temperature for 16 hr.

2.5.4 Purification of DNA Fragments from Agarose

(i) Electroelution

DNA was purified from agarose gels by cutting out (with a razor blade) gel slices containing an ethidium bromide stained DNA band. Gel slices were placed into dialysis tubing with 0.5 to 1.0 ml of 0.25 X TBE and the whole piece of tubing placed into an electrophoresis tank containing the same buffer. The DNA fragment was electroeluted from the gel slice at 100 V (20 mA) for 3 hr. The gel slice was discarded and the buffer

within the tubing was extracted three times with an equal volume of a mixture containing phenol: chloroform: isoamyl alcohol (in the ratio 25:24:1 and the phenol had been equilibrated against TE, pH 8). The two phases were separated after each extraction by a 10 min. centrifugation in an eppendorf centrifuge and the aqueous phase was transferred to a clean tube for a repeat extraction. The DNA was finally recovered by a standard ethanol DNA precipitation method (2.4.2) and resuspended in 50 µl of TE.

(ii) Ultracentrifugation

The ethidium stained band of DNA was cut out of an agarose gel, placed in a Beckman thick wall polycarbonate tube (1 ml, #343778) and TE added such that the total volume was 1 ml. Samples were balanced (to within 1 mg) and centrifuged in a swinging bucket, Beckman TLS55 rotor, at 55,000 rpm for 2 hr at room temperature with no brake. After centrifugation, the supernatant was recovered and extracted three times with phenol-chloroform by the method described in 2.5.4.i. The DNA was ethanol precipitated by standard methods (2.4.2) and finally resuspended in 50 µl of TE.

2.5.5 Transfer of DNA to Nylon Membranes (Southern Blot)

This protocol for capillary blotting of DNA from agarose gels is derived from that described by Southern (1975), which has been modified by the Amersham Corp. (1985). Restricted DNA fragments were displayed by horizontal agarose gel electrophoresis (2.5.1) and after photography, it was soaked in 0.25 M HCl for 10 min. with gentle agitation and one change of solution after 5 min. The gel was rinsed with distilled water and soaked in denaturing buffer (1.5 M NaCl, 0.5 M NaOH) for 40 min. with gentle agitation. The gel was again rinsed with distilled water and soaked in neutralization buffer (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM Na_2EDTA) for 15 min. with gentle agitation. The gel was then soaked for 2 min. in 2 X SSC buffer (20 X stock is 3.0 M NaCl and 0.3 M Na_3-citrate) and then placed upside-down on the blotting

apparatus (Maniatis *et al.*, 1982, p 385). The apparatus was: two separated tanks, filled with 20 X SSC, linked by a Whatman 3MM blotting paper wick that passes over a perspex tray, which is slightly larger than the agarose gel, and the whole wick system covered with plastic wrap except for a rectangular window with dimensions matching those of the gel to be blotted. The gel was placed on top of this blotting paper, over the window, followed by a sheet of Hybond-N™ nylon membrane (Amersham Corp.), layered carefully in a single motion over the surface of the gel. Three sheets of 3MM paper, wetted in 2 X SSC, were placed on top of the nylon membrane, followed by a 5 cm stack of absorbant paper towels, then a sheet of perspex slightly larger than the paper towels, and finally a 500 g weight on the top. The DNA was blotted onto the nylon membrane for 16 hr, the apparatus was then disassembled and the membrane rinsed in 2 X SSC for 30 sec. and air dried. The DNA was cross-linked to the nylon membrane by wrapping the dried membrane in plastic wrap and placing it DNA-side down over a 302 nm ultraviolet transilluminator for 4 min. The DNA is now permanently fixed to the membrane and after removal of the plastic, it is ready for probing by hybridization (see 2.6.1).

An alternative blotting procedure for agarose gels containing restricted plasmid DNA, where the target sequences were therefore abundant, produced two mirror image blots by the following procedure. After preparing the agarose gel by the method described above, instead of placing the gel on the blotting apparatus, it was instead placed on a sheet of perspex and here a sheet of nylon membrane was layered against the gel. Six dry sheets of 3MM blotting paper were then layered on top of the membrane, followed by a 4 cm stack of absorbant paper towels. The whole system was inverted on the bench, with care not to disturb the interface between the nylon and the gel, the perspex sheet was removed to expose the other side of the gel and another nylon membrane was placed against this gel surface. Again, six sheets of 3MM paper were placed on top of the nylon, followed by a 4 cm stack of paper towels and the perspex sheet placed on the very top in order to

keep the system flush and gently compressed. The DNA blotted out in both directions over 16 hr and the nylon membranes were subsequently treated in the same way as those described above.

2.5.6 Colony and Plaque Blotting onto Nitrocellulose

This procedure is based on a method described by Benton and Davis (1977). A nitrocellulose filter disc (0.45 µm pore size), cut to a diameter 5 mm less than the diameter of the petri dish, was layered carefully in a single motion on top of the colonies or plaques to be screened. Orientation marks were placed on the disc and plate for later identification of positive clones. After 1 min. the filter disc was then removed from the plate, again in a single motion, and placed colony side up on Whatman 3MM blotting paper saturated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 10 min. The filter disc was then transferred to another sheet of 3MM paper saturated with neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 1 mM Na_2EDTA) for 5 min. and then this neutralization step again repeated for 5 min. The filters were then washed in 2 X SSC for 30 sec. and then air dried for 30 min. The DNA was fixed to the nitrocellulose by baking dried filter discs at 80°C under a vacuum for 2 hr. The discs were now ready for hybridization.

2.6 Hybridization Procedures

2.6.1 DNA Hybridization Conditions

The following is the method by which radioactively labelled DNA probes (2.4.5) were annealed to DNA fixed on solid membrane supports (Amersham handbook, 1985). Nylon or nitrocellulose filters (2.5.5 and 2.5.6) were heat sealed in plastic bags (2 filters per bag) containing 15 ml of hybridization buffer. The hybridization buffer contains 5 X SSPE (20 X stock is 3.6 M NaCl, 20 mM EDTA, 200 mM sodium phosphate buffer, pH 7.7), 5 X Denhardt's solution (100 X stock is 2% bovine serum albumin [BSA], 2% polyvinyl pyrollidone [PVP], 2% Ficoll), 0.5% SDS and 20 $\mu g.ml^{-1}$ of freshly denatured (boiled at 100°C), sonicated calf thymus DNA. The plastic bags,

containing filters and hybridization buffer, were pre-hybridized by incubating at 67°C for 16 hr (Southern blots of genomic DNA) or 4 hr (Southern blots of plasmid DNA) or 1 hr (colony/plaque blots). The hybridization reaction begins when the denatured radioactive probe DNA (2.4.5) was added to the hybridization mixture. Hybridization was continued for 16 hr at 67°C (for homologous sequences) or lower temperatures for non-homologous related sequences (usually 50 or 55°C). Following hybridization, the membranes were washed in 2 X SSC (20 X stock is 3.0 M NaCl and 0.3 M Na_3-citrate) for 90 min. with shaking, at room temperature and with changes of the buffer every 10 min. For homologous sequences a final high stringency wash was included; 0.1 X SSC at 55°C for 10 min. The excess washing buffer was dripped off the membranes and while still damp, they were wrapped in plastic wrap and autoradiographed at -90°C, with the X-ray film between intensifying screens (DuPont).

For hybridizations involving kinased oligodeoxynucleotides (2.4.4), the hybridization buffer was 50% formamide, 5 X SSPE, 5 X Denhardt's solution, 0.5% SDS and 20 $\mu g.ml^{-1}$ of freshly denatured sonicated calf thymus DNA. The membranes were prehybridized at 4°C for 24 hr and hybridized at 4°C for 48 hr. Following hybridization, the membranes were washed in 2 X SSC for 60 min. with shaking, at room temperature and with changes of the buffer every 10 min. All other details are the same as those described above.

2.6.2 Removal of Probe DNA from Nylon Membranes

DNA blots on nylon filters could be reprobed after the removal of earlier hybridized probe DNA. This was accomplished by incubating the membrane in 400 mM NaOH at 80°C for 45 min. with shaking. The membrane was then transferred to another solution (0.1 X SSC, 0.1% SDS, 200 mM Tris-HCl, pH 7.5) and incubated at 80°C for 45 min. with shaking. Finally the membrane was rinsed in 2 X SSC at room temperature

and it was then ready for hybridization with another probe. The extent to which the labelled probe had been removed was sometimes checked by autoradiography.

2.7 Cloning and Transposon Mutagenesis Procedures

2.7.1 Preparation of Competent *E. coli* Cells

The method for preparing competent *E. coli* cells was a modification of that described by Cohen *et al.* (1972) and all steps in the procedure were aseptic. A single colony of the *E. coli* recipient strain (usually NM522 or JM107) was inoculated into 5 ml of liquid LB media and grown at 37°C for 16 hr with shaking. This was then subcultured by inoculating 200 µl of this dense culture into 100 ml of LB liquid media. The subculture was incubated at 37°C with shaking until the optical density at λ=600 nm (OD_{600}) was approximately 0.5, which usually required a 2 hr incubation for *recA*$^+$ strains or 2.5 hr for *recA*$^-$ strains. The cells were chilled on ice for 5 min. and then centrifuged in an SS34 rotor (4 tubes) at 5000 rpm, 4°C, for 10 min. The supernatant was discarded and the pelleted cells (kept on ice) were resuspended and combined in a total of 25 ml of 100 mM $MgCl_2$ (ice cold). Resuspension at each step was by drawing the cells slowly up and down using a pipette and not by vortexing. The cells were again centrifuged in an SS34 rotor (pooled into 1 tube) at 5000 rpm, 4°C, for 10 min. The pelleted cells (on ice) were gently resuspended in 25 ml of 100 mM $CaCl_2$ (ice cold) and incubated on ice for 40 min. The cells were again centrifuged in an SS34 rotor at 5000 rpm, 4°C, for 10 min. and then resuspended (on ice) in 5 ml of 100 mM $CaCl_2$ (ice cold). The cells were now competent for the uptake of DNA by the transformation procedure (2.7.2) and were stored for indefinite periods at $^-$90°C in 15% glycerol. A 0.9 ml volume of 100% glycerol was mixed in to the suspension of competent cells, which was then divided into 300 µl aliquots, snap frozen in liquid N_2, and stored at $^-$90°C.

2.7.2 Transformation of Competent *E. coli* Cells

An aliquot of frozen competent cells (2.7.1) was thawed slowly on ice for 15 min. Ligation mixes (2.4.3) or plasmid DNA were added to the thawed competent cells and the mixture incubated on ice for 60 min. The transformation mixture was then heatshocked for 90 sec. in a 42°C water bath and then 2 ml of liquid LB (at 37°C) was added. The transformed cells were then incubated at 37°C without shaking for 1 hr (if antibiotic marker was ampicillin or kanamycin) or 2 hr (if antibiotic marker was chloramphenicol, or tetracycline). The transformants were plated out on LB solid media supplemented with the appropriate antibiotic and incubated at 37°C for 16 hr. When derivatives of pUC plasmids were utilized as the cloning vector, 5 mM IPTG (isopropyl-β-D-thiogalacto-pyranoside) and 40 $\mu g.ml^{-1}$ X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside dissolved in dimethyl formamide) were incorporated into the molten LB media before pouring. Colonies carrying recombinant plasmids were unable to cleave the galactosyl residue from the synthetic X-gal substrate and consequently formed clear colonies, while those transformants carrying vector (without insert) formed blue colonies.

2.7.3 Transfection of *E. coli* by M13 Bacteriophage DNA

This method is initially the same as for plasmid DNA transformations (2.7.2). The ligation reactions were added to thawed competent *E. coli* cells, mixed thoroughly and incubated on ice for 30 min. The mixture was heatshocked at 42°C for 90 sec. and aliquots (1, 10 and 100 µl) were added to a solution comprising: 100 µl of fresh dense JM107 culture, 10 µl of X-gal (0.1 $mg.ml^{-1}$ in dimethyl formamide), 20 µl of 100 mM IPTG, 3 ml of soft (0.75% agar) molten LB media (approx 45°C). This mixture was rapidly mixed and overlaid on top of solid LB media, then incubated at 37°C for 16 hr. Recombinant phage generally produce colorless plaques and M13 non-recombinants form blue plaques.

2.7.4 Transposon Mutagenesis with Mu dII-1734

The following method was published by Bassam *et al.* (1988) and makes use of the transposon Mu dII-1734 (Castilho *et al.*, 1984). This transposon carries a promoterless *E. coli lacZYA* operon and upon insertion, it can create translational fusions of the promoterless *lac* genes with the target gene. The plasmid pJG100 was transformed (2.7.2), omitting the 42°C heatshock step, into *E. coli* strain POII1734, which harbours the Mu dII-1734 transposon and a temperature sensitive helper Mu in its chromosome. Strain POII1734 and its transformants were never grown above 28°C, because at higher temperatures Mu dII-1734 will readily transpose. Transformant strain POII1734-(pJG100) was inoculated into 5 ml of liquid LB + 10 mM $MgCl_2$ and cultured for 16 hr at 28°C with shaking. A 70 µl volume of the dense culture was subcultured into 5 ml of liquid LB + 10 mM $MgCl_2$ and grown at 28°C with shaking (approximately 5 hr) until the culture was in log phase ($OD_{600} \approx 0.5$). A 1 ml aliquot of these freshly grown cells was incubated at 43°C for 30 min. and at this temperature the Mu dII-1734 was induced to transpose by the helper Mu phage. After the 30 min. transposition period, the 1 ml tube of cells was incubated at 37°C for 2 hr. A 20 µl volume of chloroform was added, the cells were vortexed for 10 sec. to aid lysis, and the sample was centrifuged (eppendorf) for 1 min. A 100 µl volume of the supernatant (containing Mu particles) was added to 1 ml of fresh stationary phase *E. coli* strain JM107 grown in liquid LB + 5 mM $MgCl_2$ + 10 mM $CaCl_2$. The mixture was incubated at 28°C with out shaking for 20 min. to allow preabsorption of the phage and then 9 ml of liquid LB was added and the mixture was incubated for a further 45 min. at 28°C. Transfected cells were plated out on solid LB media, supplemented with 50 $\mu g.ml^{-1}$ chloramphenicol, 50 $\mu g.ml^{-1}$ kanamycin, 0.4 $mg.ml^{-1}$ X-gal, and incubated at 37°C for 16 hr.

CHAPTER THREE

Cloning and Mapping of *exo* Loci

3.1 INTRODUCTION

The cloning and physical characterization of wild-type DNA from the *exo* region would facilitate investigations of gene organization and regulation of polysaccharide synthesis in strain NGR234. From a previous study (Chen *et al.*, 1988) it was observed that an R-prime plasmid (R'3222) carrying approximately 64 kb of wild-type NGR234 DNA, was able to restore an Exo^+ phenotype to 28 out of 30, Tn*5* induced, Exo^- mutants (group 2 classification, Chen *et al.*, 1985). This indicated that the mutated loci in the 28 Exo^- mutants were physically linked to this cloned region of DNA. Separate R-prime plasmids carrying the different Tn*5* mutated *exo* loci were constructed and used in complementation tests; six complementation classes (A to F) were identified (Chen *et al.* 1988). Two genetic groups that had an unusual phenotype were those of genetic groups E and F. Mutants at these loci were Exo^- and their phenotype could be corrected to Exo^+ by R'3222. However, derivatives of R'3222 that carry Tn*5* insertions at the E and F locations, confer an Exo^- phenotype on the wild-type strain ANU280, which is normally Exo^+. From this it was concluded that the episomally carried allele was dominant and the dominance was a result of copy number for that allele. The dominance of the mutant loci from genetic group F, when carried on R-prime plasmids was persistent (ie. remained indefinitely Exo^-), while the dominance associated with genetic group E was leaky (ie. produced slightly mucoid colonies after prolonged incubation periods). The model proposed to explain these results, suggested that Tn*5* insertions in this gene resulted in the generation of "dominant negative mutations" (reviewed by Herskowitz, 1987), which led to the functional inactivation of EPS production in the wild-type strain. It was speculated that a truncated but bioactive gene product was still produced after the insertion of Tn*5* into the gene, and that the truncated gene product was a fragment of a *trans*-acting factor that competes for the same regulatory promoters as the wild-type gene

products, but fail to activate the expression of the target gene. It was also considered that the wild-type gene products may form a multimeric regulatory protein and the interaction of the truncated gene products yields an inactive protein.

The objectives of the experiments detailed in this chapter were to subclone the *exo* genes, with an emphasis on the group E and F loci and then to investigate the genetic organization of *exo* genes in the vicinity of these mutations.

3.2 RESULTS

3.2.1 Cloning of the Wild–Type Allele for the 2811 Locus (*exoY*)

Recombinant plasmid pHC11 has a 6.5 kb *Eco*RI restricted DNA fragment carrying the Tn*5* transposon from the Exo$^-$ mutant strain ANU2811 (Chen *et al.*, 1988). The insert from this clone was radioactively labelled and used to probe wild-type, strain ANU280, genomic DNA and R'3222 DNA that had been restricted with a variety of suitable cloning endonucleases. After Southern blot hybridization, a hybridizing *Bam*HI DNA fragment of 10 kb in size was considered desirable for cloning. This wild-type fragment was cloned from R'3222 DNA into the vector pUC18, by screening for *E. coli* strain JM107 recombinant clones that hybridized to the insert DNA of pHC11. The 10 kb *Bam*HI fragment was cloned in both orientations to generate plasmids named pJG11 and pJG12. Verification that pJG11 was a clone carrying wild-type DNA from the 2811::Tn*5* insertion site is provided by Fig. 3.1. The hybridization patterns of R'3222 and R'2811 (R'3222 carrying a Tn*5* at the 2811 site), alongside the genomic DNA for strains ANU280 and ANU2811, were all identical except for the fragment where the Tn*5* had inserted (Fig. 3.1). In strain ANU2811 the Tn*5* had inserted into a 0.6 kb *Eco*RI restriction fragment and thus increased the size of this fragment to 6.5 kb. A partial restriction map of the pJG11 insert DNA is provided in Fig 3.2.

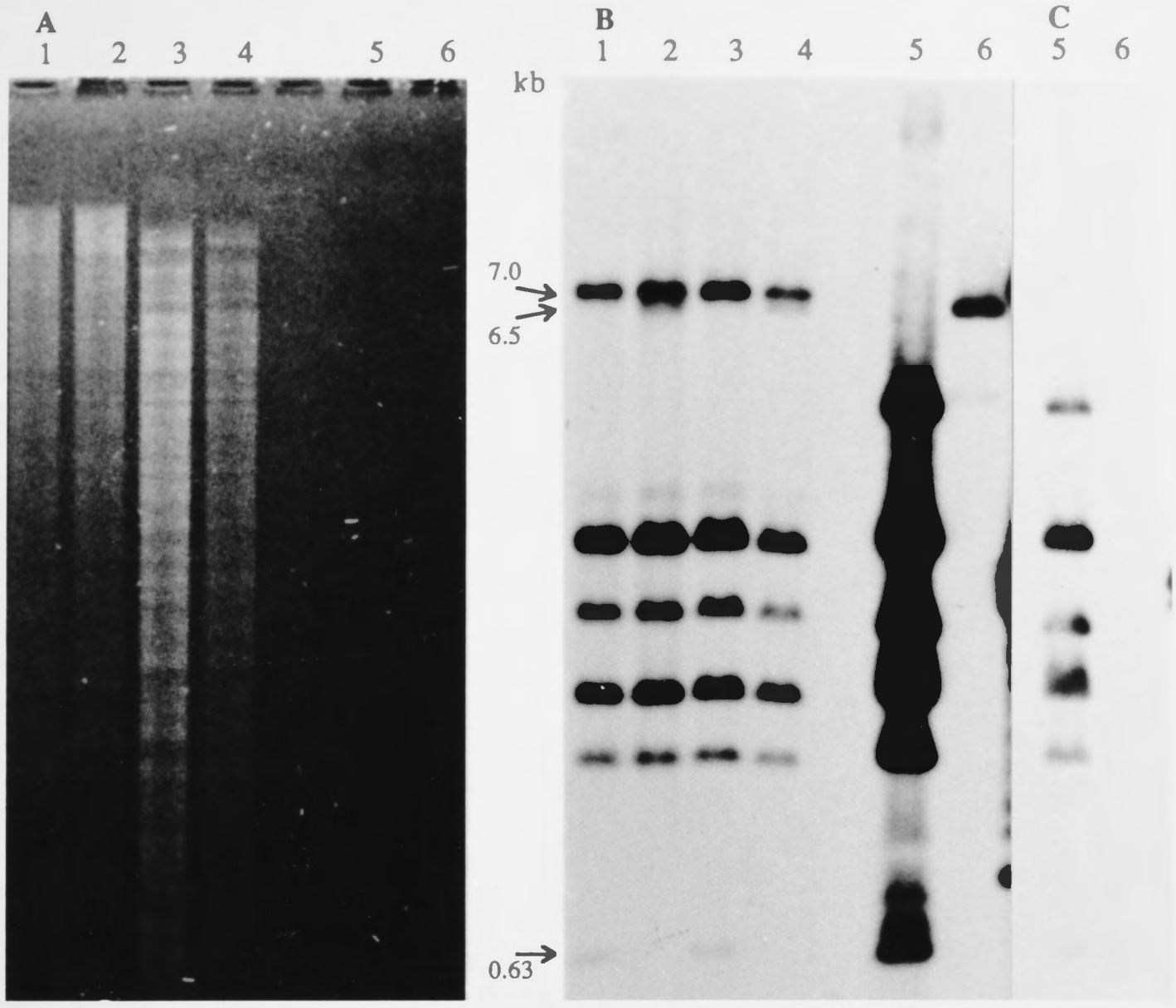


Fig. 3.1 Cloning of wild-type DNA flanking the 2811::Tn*5* insertion site.

(**A**) Electrophoresis of *Eco*RI restricted DNA through a 0.7% agarose gel.
Lane 1; genomic DNA from *E. coli* strain HB101 carrying R'3222.
Lane 2; genomic DNA from *E. coli* strain HB101 carrying R'2811.
Lane 3; genomic DNA from ANU280
Lane 4; genomic DNA from ANU2811
Lane 5; pJG11 plasmid DNA
Lane 6; pHC11 plasmid DNA

Varying amounts of DNA were deliberately loaded into certain wells; lanes 3 and 4 have approximately three times more DNA than in lanes 1 and 2, to compensate for the variation in amounts of target sequences. For the same reason, very small amounts of plasmid DNA were electrophoresed through lanes 5 and 6; these low concentrations could not be detected by ethidium bromide staining.

(**B**) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled 10 kb *Bam*HI insert DNA from pJG11. The wild-type DNA is characterized by the presence of a 0.63 kb *Eco*RI fragment and the Tn*5* mutated DNA is characterized by a 6.5 kb *Eco*RI fragment.

(**C**) Part of the same autoradiograph as in panel B, that was exposed for less time to more clearly show the hybridization pattern of DNA cloned into pJG11.

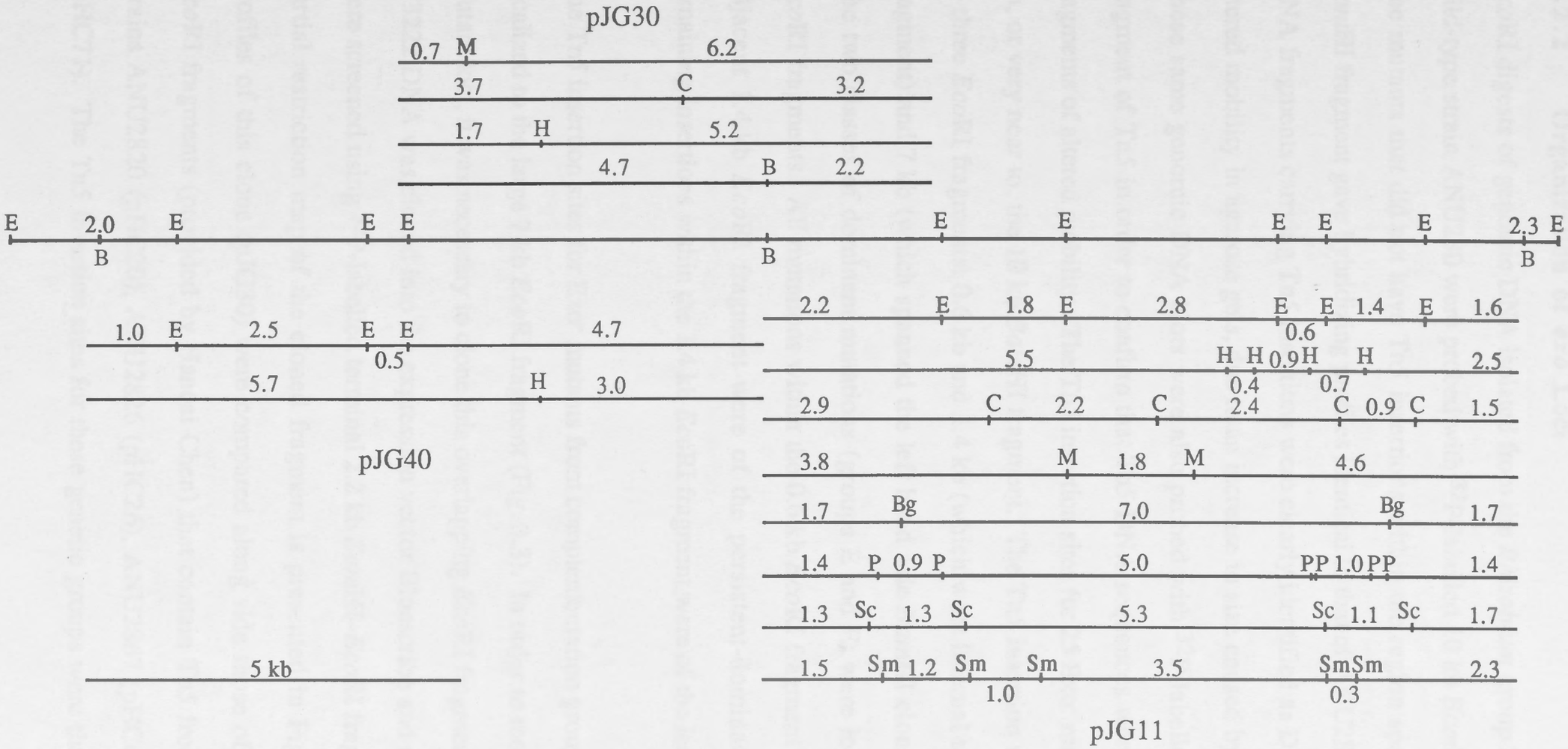


Fig. 3.2 Partial restriction map of cloned *exo* region.

Three overlapping cloned fragments (pJG11, pJG30 and pJG40) were used to construct the map. A separate map for each clone is provided for each restriction endonuclease . The numbers refer to the sizes in kb of the restricted fragments. The sizes of the terminal *Eco*RI fragments are also indicated, as judged from hybridization of restricted ANU280 DNA to probes of the region. Restriction sites are: B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; P, *Pst*I; Sc, *Sac*I; Sm, *Sma*I.

3.2.2 Organization of *exo* Loci

*Eco*RI digests of genomic DNA isolated from the *Rhizobium* group 2 Exo$^-$ mutants and wild-type strain ANU280 were probed with ^{32}P-labelled 10 kb *Bam*HI insert of pJG11. The mutants that did not have Tn*5* insertions within the region spanned by the 10 kb *Bam*HI fragment gave hybridizing profiles identical to that of ANU280. Genomic *Eco*RI DNA fragments carrying Tn*5* insertions were clearly identified as DNA fragments with altered mobility in agarose gels, due to an increase in size caused by the Tn*5* insertion. These same genomic DNA blots were also probed with ^{32}P-labelled internal *Hin*dIII fragment of Tn*5* in order to confirm that Tn*5* DNA sequences were also within DNA fragments of altered mobility. The Tn*5* insertion sites for 25 Exo$^-$ mutants were mapped on, or very near to, the 10 kb *Bam*HI fragment. The Tn*5* insertion sites were localized to three *Eco*RI fragments: 0.6 kb and 1.4 kb (which were internal to the 10 kb *Bam*HI fragment) and 7 kb (which spanned the left hand side *Bam*HI cloning site) (Fig. 3.3). The two classes of dominant mutations (groups E and F) were localized to adjacent *Eco*RI fragments. All mutations within the 0.6 kb *Eco*RI fragment and two within the adjacent 1.4 kb *Eco*RI fragment were of the persistent-dominant class, while the remaining insertions within the 1.4 kb *Eco*RI fragment were of the leaky-dominant class.

The Tn*5* insertion sites for Exo$^-$ mutants from complementation groups A, B and D were localized to the large 7 kb *Eco*RI fragment (Fig. 3.3). In order to more finely map these mutant loci, it was necessary to clone this overlapping *Eco*RI fragment. *Eco*RI restricted R'3222 DNA was cloned into the expression vector Bluescribe and recombinant clones were screened using ^{32}P-labelled terminal 2.2 kb *Bam*HI–*Eco*RI fragment of pJG11. A partial restriction map of the cloned fragment is presented in Fig. 3.2. Restriction profiles of this clone (pJG30) were compared along side those of previously cloned *Eco*RI fragments (provided by Hancai Chen) that contain Tn*5* from the Exo$^-$ mutant strains ANU2820 (pHC20), ANU2826 (pHC26), ANU2867 (pHC67), and ANU2871 (pHC71). The Tn*5* insertion sites for these genetic groups were thus mapped to more

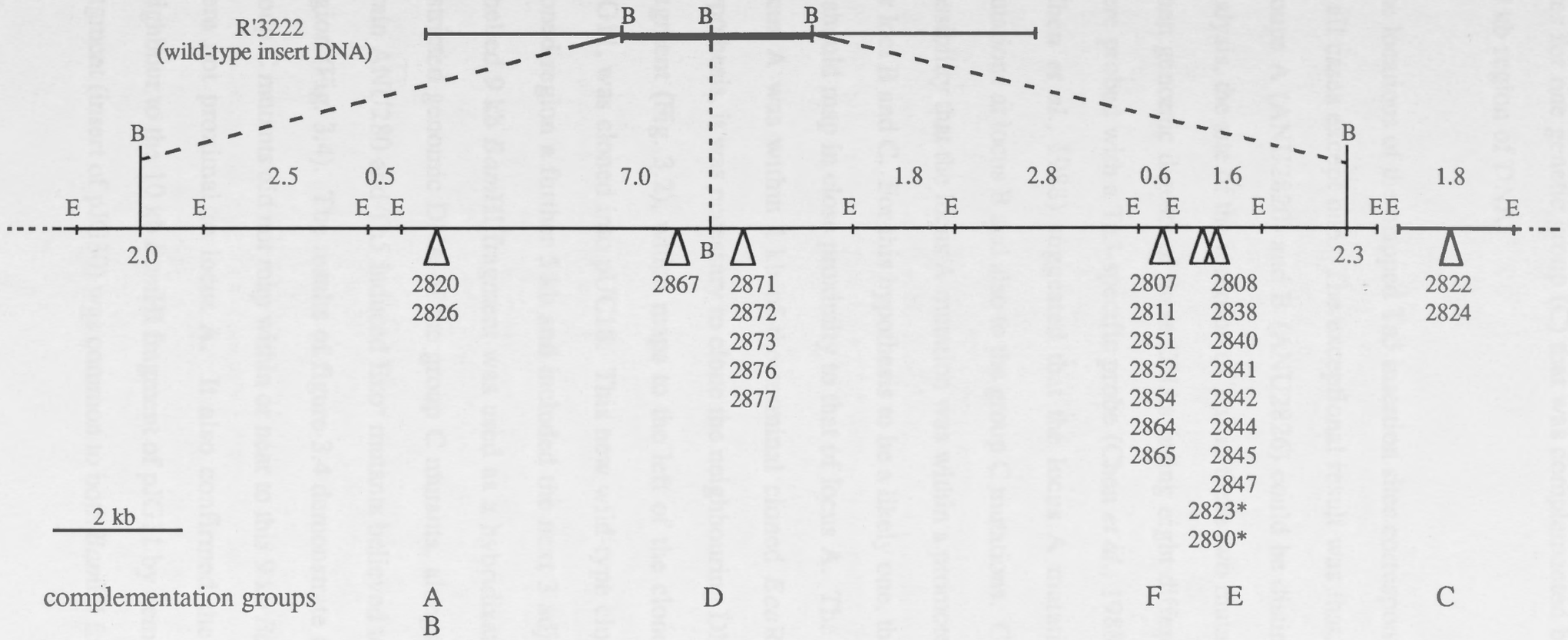


Fig. 3.3 Mapped sites of Tn*5* insertions within *exo* genes.

The locations of Tn*5* insertion sites for all group 2 Exo$^-$ mutants that were corrected by R'3222 are shown. The mutants were classified into distinct genetic groups A–F (H. Chen, 1987, Ph. D. Thesis). Restriction sites indicated are: B, *Bam*HI; E, *Eco*RI. Mutations indicated by asterisks belong to genetic group F.

defined regions within this larger 7 kb *Eco*RI fragment (Fig. 3.3). The Tn*5* insertion sites for one genetic group (C) that was complemented by R'3222, did not map in this 20 kb region of DNA.

The locations of the mapped Tn*5* insertion sites corresponded to separate genetic groups in all cases except one. The exceptional result was that, while the mutants in genetic groups A (ANU2820) and B (ANU2826) could be distinguished by complementation analysis, the site of the insertion of Tn*5* in these two mutants could not be distinguished when genomic digests of these DNAs (using eight different restriction endonucleases) were probed with a Tn*5*-specific probe (Chen *et al.*, 1988). The complementation data (Chen *et al.*, 1988) suggested that the locus A mutation was *cis*-dominant to the mutations at locus B and also to the group C mutations. Chen *et al.* (1988) discussed the possibility that the locus A mutation was within a promoter that operated bidirectionally for loci B and C. For this hypothesis to be a likely one, the Tn*5* insertion sites for group C should map in close proximity to that of locus A. The mapped Tn*5* insertion site for locus A was within 1 kb of the terminal cloned *Eco*RI site. Therefore to test the hypothesis, it was necessary to clone the neighbouring DNA fragments. A 9 kb *Bam*HI fragment (Fig. 3.2), which maps to the left of the cloned 10 kb *Bam*HI fragment of pJG11, was cloned into pUC18. This new wild-type clone (pJG40) now extended the cloned region a further 5 kb and included the next 3 adjacent *Eco*RI fragments. ^{32}P-labelled 9 kb *Bam*HI fragment was used as a hybridization probe for blots of *Eco*RI restricted genomic DNA of the group C mutants, alongside *Eco*RI restricted DNA of strain ANU280 and Tn*5* induced Exo$^-$ mutants believed to map within, and outside, the region (Fig. 3.4). The results of figure 3.4 demonstrate that the Tn*5* insertions for the group C mutants did not map within or near to this 9 kb *Bam*HI fragment and hence they were not proximal to locus A. It also confirmed the fragment was the authentic neighbour to the 10 kb *Bam*HI fragment of pJG11 by demonstrating that the 7 kb *Eco*RI fragment (insert of pJG30) was common to both *Bam*HI fragments.

Fig. 3.4 Directional cloning of *exo* DNA and mapping of Tn*5* insertions.
(**A**) Electrophoresis of *Eco*RI restricted genomic DNA through a 0.7% agarose gel. Strains are described in table 2.1 and in figure 3.3.

Lane 1; ANU2811, Lane 2; ANU280,
Lane 3; ANU2820, Lane 4; ANU2822,
Lane 5; ANU2826, Lane 6; ANU2824,
Lane 7; ANU2867, Lane 8; ANU2871,
Lane 9; ANU2822, Lane 10; ANU280,
Lane λ; Lambda DNA digested with *Hin*dIII.

Equal amounts of DNA were not necessarily loaded into each well and the DNA in lanes 4 and 7 appears to be partially degraded.

(**B**) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled 9 kb *Bam*HI insert DNA from pJG40. The DNA fragments entirely homologous to the cloned DNA are indicated with arrows. The cloned DNA is obviously reiterated at another site within the genome as evidenced by the presence of two other hybridizing fragments. Tn*5* containing fragments are characterized by their absence and corresponding replacement by a larger fragment.

In order to further address the dilemma as to whether the mutation at locus A had interrupted the promoter acting on locus B, the actual Tn*5* insertion sites were sequenced. The two mutant loci for strains ANU2820 and ANU2826 shared identical Tn*5* insertion sites, yet they fall into different complementation classes. Therefore, the most appropriate interpretation of the data is that complementation class A was a double mutant at loci B and C. Mutant ANU2820 (A) has a single Tn*5* insertion at the same site as ANU2826 (B) and it also appears to have a secondary point mutation in the same locus as group C.

3.3 DISCUSSION

Presented in this chapter is the physical characterization of a 20 kb region of wild-type DNA known to contain genes responsible for EPS biosynthesis. It was previously known that Exo$^-$ mutants from six complementation groups could be corrected by the introduction of a large R-prime plasmid carrying an estimated 64 kb of *Rhizobium* DNA (Chen *et al.*, 1988). The region within this R-prime carrying the *exo* genes has been subcloned as overlapping fragments of manageable size for further molecular investigation. The locations of insertion sites for Tn*5* in each of the mutants spanned by this region were determined molecularly by restriction and hybridization analysis. The loci for five of the six genetic groups are clustered within a 15 kb region of cloned DNA. While the locus for the remaining genetic group (C) was shown to map outside the cloned region, it is believed to map proximally to the right (Fig. 3.3), (discussed in chapter 6).

The molecular relationship between Exo$^-$ mutants of groups A, B and C was investigated. It was shown that *exo* locus C was not located proximally to that of *exo* loci A or B and and that *exo* loci A and B had the same Tn*5* insertion site. This dispelled the hypothesis that the *exo* locus A mutation was in fact within a bidirectional promoter controlling separate genes at *exo* loci B and C. The only reasonable interpretation, is that

the mutant of genetic group A is in fact be a double mutant with a Tn*5* insertion at the same location as that for the group B mutant and a further secondary mutation, not involving Tn*5*, at *exo* locus C.

It was known previously (Chen *et al.*, 1988) from analysis of restricted mutant genomic DNA that two insertion site locations existed for complementation group D. After mapping the locations for these Tn*5* insertion sites to their relative positions on the restriction map, their close linkage was demonstrated. Similarly, the Tn*5* insertion sites for the two dominant genetic groups were previously known to be inserted within different *Eco*RI fragments. Following these mapping studies the two *Eco*RI fragments were shown to be adjacent. The mutant *exo* alleles at loci F and E gave a persistent and leaky dominant phenotype, respectively, to the wild-type alleles, only when the mutated alleles were present at an elevated copy number with respect to the corresponding wild-type allele (Chen *et al.*, 1988). Since the Tn*5* insertions for the persistent and leaky dominant classes were physically adjacent, it was not clear whether this represented two separate genes or Tn*5* insertions in two separate sites of the same gene. The insertion of Tn*5* into either of these two sites may result in the production of a truncated, but bioactive gene product; albeit with a different bioactivity than the wild-type product. The leaky dominance of locus E mutant alleles may indicate a low level of wild-type function associated with mutant gene products that are not as severely truncated as gene products resulting from locus F mutations. Alternatively, loci F and E may represent separate genes with different bioactive functions, which then results in their truncated gene products causing slightly different phenotypes. Cloning of the *exo* genes and determining their genetic organization has supported and advanced upon their classification by genetic complementation alone. In addition, the information now permits further investigations into their biological functions and modes of regulating EPS production in *Rhizobium*.

Publication

The information presented in this chapter was part of my contribution to the publication:-

Chen, H., J. X. Gray, M. Nayudu, M. A. Djordjevic, M. Batley, J. W. Redmond, and B. G. Rolfe. (1988). Five genetic loci involved in the synthesis of acidic exopolysaccharides are closely linked in the genome of *Rhizobium sp* strain NGR234. *Mol. Gen. Genet.* **212**:310-316.

Acknowledgement

The clones and information provided by Hancai Chen is appreciated and has been properly referenced where appropriate.

CHAPTER FOUR

DNA Sequences and Resultant Phenotypes of *exoX* and *exoY*

4.1 INTRODUCTION

In *R. leguminosarum* bv. *phaseoli*, the involvement of three genes, termed *psi*, *psr* and *pss*, in EPS biosynthesis has recently been demonstrated (Borthakur and Johnston, 1987, Borthakur *et al.*, 1985, 1986 and 1988). Mutant strains defective in *psi* induce Fix$^-$ nodules. When multiple copies of *psi* were introduced on a plasmid into *R. l.* bv. *phaseoli*, both EPS synthesis and nodulation ability were inhibited. Genes *psr* and *pss* could counter the inhibitory effects of *psi* when either one of *psr* or *pss* was present at an equivalent copy number to *psi*. Strains that are *psr*::Tn*5* mutants were still capable of EPS production, albeit at reduced levels compared to the wild-type, but *pss*::Tn*5* mutants were completely Exo$^-$. This suggested that *pss* is probably the principle factor involved in counter-acting the effects of *psi*. Both *psi* and *psr* are separated by 13 kb and are both located on the symbiotic megaplasmid, whereas the *pss* operon is not located on the symbiotic megaplasmid. The nucleotide sequence of *psi* showed that it specified a polypeptide of 86 amino acids with a hydrophobic N-terminal region spanning 41 amino acids. This suggested that the *psi* product was associated with the cell membrane (Borthakur and Johnston, 1987). Psi is not the only regulatory protein involved in repression of EPS synthesis. In *R. fredii*, another gene, *nodD2*, prevents EPS synthesis when carried on a multicopy plasmid (Appelbaum *et al.*, 1988). The mechanism by which the genes *psi* and *nodD2* inhibit EPS production is unknown.

The timing and regulation of EPS synthesis may be critical for successful nodule formation. On the basis that *psi*::Tn*5* mutants of *R. l.* bv. *phaseoli* are Fix$^-$ and that *psr* was shown to transcriptionally repress *psi*, Borthakur and Johnston (1987) have hypothesized that the apparent inhibition of EPS synthesis by *R. l.* bv. *phaseoli* in the bacteroid state is a prerequisite for nitrogen fixation and is achieved by a repression of

psr, which results in a derepressed *psi*. However, in *R. meliloti* it appears that some EPS genes, which are actively transcribed during the free living state, remain strongly expressed during symbiosis (Keller *et al.* 1988). To date, it is not clear whether EPS is produced in the bacteroid state, or indeed whether the regulation of EPS production is the same for all *Rhizobium* species.

In chapter 3, molecular investigations demonstrated that five genetic loci involved in the synthesis of acidic exopolysaccharide in *Rhizobium* sp. strain NGR234 were clustered in a 15 kb region of DNA. Chen (Ph.D. Thesis, 1987) showed that the introduction into the wild-type (Exo^+) strain of R-prime plasmids containing Tn*5* insertions (mapped to genetic groups F and E) resulted in merodiploid transconjugants that were stably Exo^-. Conversely, when the corresponding wild-type allele was introduced into group F or E Exo^- mutants, the episomally located *exo* allele was dominant and stable Exo^+ transconjugants resulted. This chapter demonstrates that group F and E mutations are located within a single gene, *exoY*. In contrast to the hypothesis of Chen (Ph.D. Thesis, 1987), mutations in *exoY* do not act as dominant negative mutations. Instead, this chapter conclusively demonstrates that another *exo* gene less than 1kb from the group F and E mutation sites is responsible for the Exo^- phenotype of the merodiploid strains. This novel NGR234 *exo* gene, termed *exoX*, confers an Exo^- phenotype only when it is present in a copy number above that of *exoY* or when *exoY* had been mutated or deleted.

4.2 RESULTS

4.2.1 Transfer of the Wild–Type 10 kb *Bam*HI Fragment (*exoY*) into Group 2 Exo$^-$ Mutants

The 10 kb *Bam*HI fragment (insert of pJG11) known to contain wild-type DNA from the region of the 2811::Tn*5* insertion (section 3.2.1), was subcloned in both orientations into the broad-host range IncP1 vector pJJ016. These broad-host range recombinant plasmids were conjugated into strain ANU280 and many of the group 2 Exo$^-$ mutants (Table 4.1). Plasmids carrying the 10 kb *Bam*HI fragment in alternate orientations conferred the same phenotype and henceforth only results with the plasmid pJG22 will be reported. When pJG22 was transferred into strain ANU280, the transconjugants grew normally and the colony morphology remained Exo$^+$. The plasmid complemented only those Exo$^-$ mutants that were classified into complementation groups E and F. Interestingly, when pJG22 was transferred into the two dominant classes of mutants (E and F), transconjugants arose at a frequency of 3.4×10^{-4} per recipient strain (filter mating, section 2.2.3). The frequency of transfer was approximately 100-fold lower than for (i) the transfer of the vector (pJJ016) alone into these Exo$^-$ strains and (ii) the transfer of pJG22 or pJJ016 into strain ANU280. The class E or F Exo$^-$ mutants containing pJG22 were initially slow to appear (visible after 4 days growth as opposed to 3 days for other transconjugants), the growth rates of the colonies were not uniform, and a mixture of Exo$^+$ and Exo$^-$ colony morphologies resulted (Fig. 4.1). Both Exo$^+$ and Exo$^-$ transconjugants arose at approximately equal frequencies; 52% Exo$^+$ and 48% Exo$^-$ in the case of ANU2811(pJG22) transconjugants. Each of these colony types appeared clonal and were highly stable. Conversion from one colony morphology to another was not observed when the different cell types were recultured on selective medium plates. Although these colony types were initially slow to appear, the subsequent growth rates of these transconjugant variants were normal as compared to the wild-type strain.

Table 4.1 Complementation of Exo⁻ mutants by the 10 kb *Bam*HI fragment

COMPLEMENTATION GROUP	Exo PHENOTYPE OF TRANSCONJUGANT	CONJUGATION FREQUENCY
Wild-Type	Exo$^+$	HIGH
A	Exo$^-$	–
B	Exo$^-$	LOW
C	Exo$^-$	HIGH
D	Exo$^-$	HIGH
E	50%Exo$^+$/50%Exo$^-$	LOW
F	50%Exo$^+$/50%Exo$^-$	LOW
G	Exo$^-$	LOW
ANU2895*	Exo^{++}	LOW

The strains examined were: wild-type (ANU280), complementation group A (ANU2820), complementation group B (ANU2826), complementation group C (ANU2822 and ANU2824), complementation group D (ANU2867, ANU2871, ANU2872, ANU2873, ANU2876 and ANU2877), complementation group E (ANU2808, ANU2838, ANU2840, ANU2841, ANU2842, ANU2844, ANU2845 and ANU2847), complementation group F (ANU2807, ANU2811, ANU2823, ANU2851, ANU2852, ANU2854, ANU2864, ANU2865 and ANU2890) and complementation group G (ANU2818 and ANU2831). Note that the sites of Tn*5* insertion for mutants that make up complementation group G, did not map within the characterized *exo* DNA (Fig. 3.3) and the mutants were not corrected by R'3222 (Chen, Ph.D Thesis, 1987). Since the complementation properties for plasmids pJG20 and pJG22 were the same for all strains examined, there is no distinction made between them in the table. An Exo$^+$ colony is as mucoid as those of ANU280 and an Exo$^-$ colony is as dry as those of ANU2811. A "LOW" conjugation frequency refers to an approximate 10^{-2} reduction in the number of transconjugants arising from the conjugation attempt relative to the number when the frequency is "HIGH". The comparison is only made when the conjugal transfers were performed simultaneously.

* ANU2895 is not a group 2 Exo$^-$ mutant, but is instead a Tn*5* induced mutant of ANU280 that now greatly over produces EPS and has a very mucoid (Exo^{++}) phenotype. The data is included in this table, because a reduced frequency of pJG22 transconjugation was also observed for strain ANU2895.

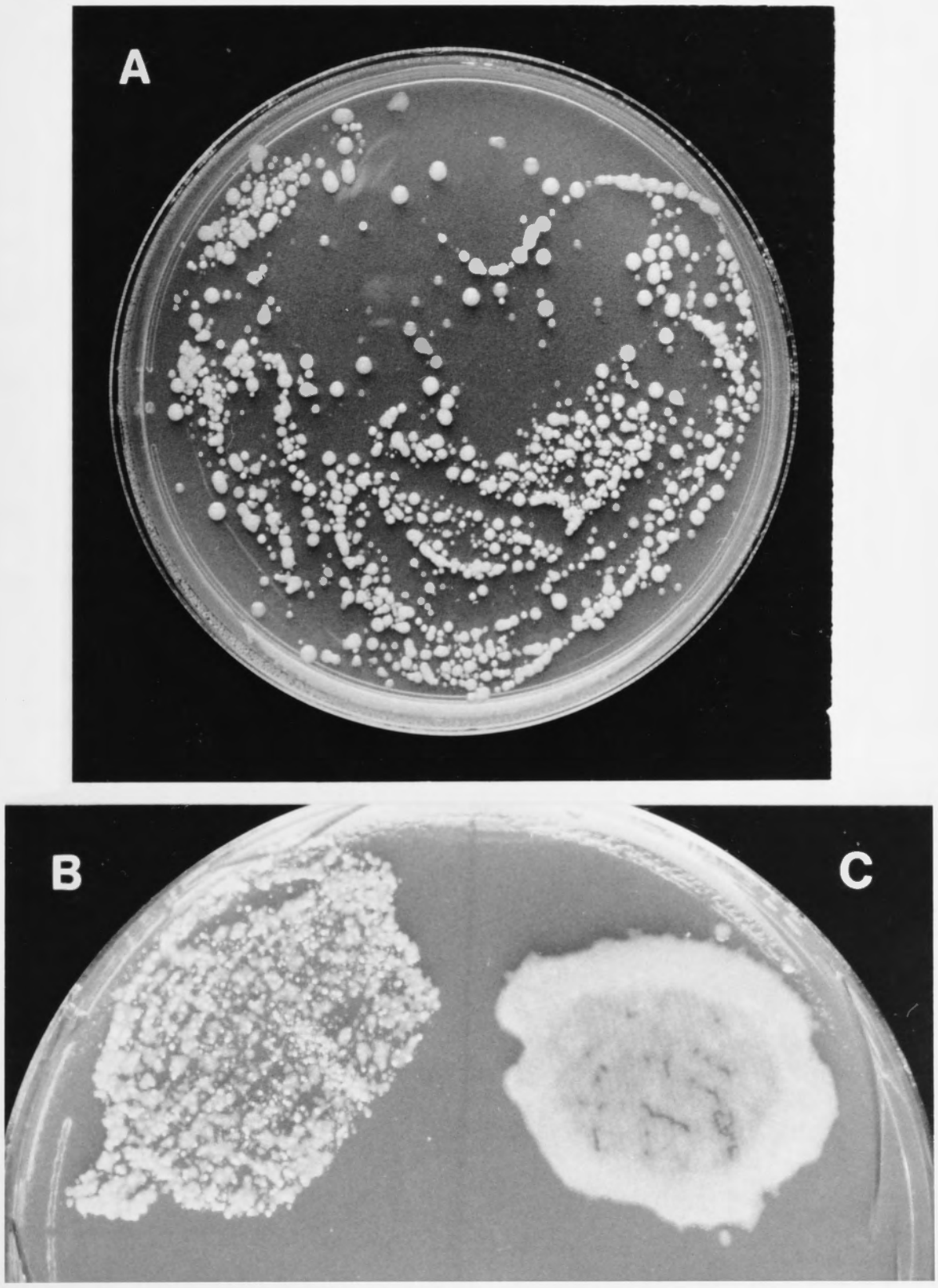


Fig. 4.1 Transconjugant phenotypes resulting from the transfer of pJG22 into strain ANU2811. **(A)** A plate of ANU2811(pJG22) transconjugants after a filter mating (2.2.3.ii), showing the variations in colony sizes (indicating non-uniform growth rates) and the two Exo phenotypes. The intense white opaque colony types are Exo^- and the more translucent colonies are Exo^+. **(B)** Resultant ANU2811(pJG22) transconjugant colony types after a patch mating (2.2.3.i) showing again both colony types and also qualitatively demonstrating the reduced frequency of transconjugation. **(C)** Resultant Exo^+ colony morphology of ANU2811(R'3222) transconjugants after a patch mating.

The reduced frequency of transconjugants was not restricted to conjugations involving the Exo$^-$ mutants of complementation groups E and F. It was also observed for Exo$^-$ mutants in complementation groups B and G, and another mutant background, ANU2895 (Table 4.1). Strain ANU2895 is a Tn*5* induced mutant of ANU280, that greatly overproduces EPS and the very mucoid phenotype is referred to as Exo^{++} (Chen, Ph.D. Thesis,1987). Normal transconjugation frequencies with pJG22 were observed for complementation groups C and D, strains ANU280 (wild-type), ANU240 (another wild-type), ANU265 (pSym$^-$), ANU1255 (*nodD*::Tn*5*) and three other EPS overproducing Tn*5* mutants of strain ANU280 (ANU2833, ANU2861, ANU2866, these do not overproduce EPS to the same extent as ANU2895).

4.2.2 Symbiotic Phenotypes of Exo$^+$ and Exo$^-$ Transconjugants

The wild-type strain ANU280 inoculated onto *Leucaena leucocephala* forms between 5 and 23 cylindrical, pink pigmented, nitrogen fixing, nodules per plant after 5 weeks. In contrast, all Exo$^-$ mutant strains (*eg*. ANU2811) formed Fix$^-$ callus-like growths (Chen *et al*., 1985). The symbiotic phenotypes of Exo$^+$ and Exo$^-$ ANU2811(pJG22) transconjugants were examined on *Leucaena*. Exo$^+$ ANU2811(pJG22) transconjugants were able to form Fix$^+$ (*ie*. reduced acetylene) nodules that were indistinguishable from those formed by the wild-type strain. However, this was not due to a simple complementation of the mutant allele by the plasmid-borne wild-type allele. Examination of the bacteria recovered from the nodules demonstrated that, in the absence of antibiotic selection, more than 50% of the cells had lost both tetracycline resistance (vector marker) and kanamycin resistance (Tn*5* marker). This result indicated that these cells underwent recombination events and subsequent loss of plasmid during nodule passage. Similarly, the Exo$^-$ ANU2811(pJG22) transconjugants induced Fix$^+$ nodules on *Leucaena*, but all bacteria isolated from nodules were Exo$^+$ and had neither tetracycline nor kanamycin resistance markers, which were both present on the plasmid (demonstrated in 4.2.3). This result again indicated recombination and plasmid loss.

4.2.3 Molecular Analysis of Exo$^+$ and Exo$^-$ ANU2811(pJG22) Transconjugants

To investigate the cause of the two colony morphologies for ANU2811(pJG22) transconjugants, plasmid DNA was recovered from single Exo$^+$ and Exo$^-$ isolates. The restriction digest profile of the plasmids recovered from the Exo$^+$ transconjugants was identical to that of the original plasmid pJG22 (Fig. 4.2, lane 1a). In contrast, the profile of the plasmid DNA from the Exo$^-$ transconjugants was altered (Fig. 4.2, lane 1b). These plasmids had lost the 0.6 kb *Eco*RI fragment, which contained the site of Tn*5* insertion in the mutant strain ANU2811, and now had a new *Eco*RI fragment, which was larger by 5.8 kb (the length of the Tn*5* sequences). Hybridization analysis (Fig. 4.2) showed that this new 6.5 kb *Eco*RI fragment carried Tn*5*. In summary, the plasmids isolated from Exo$^-$ transconjugants were clones of the 10 kb *Bam*HI fragment with a Tn*5* insertion within the 0.6 kb *Eco*RI fragment, and are henceforth termed pJG22::Tn*5*. In contrast, the plasmids isolated from Exo$^+$ transconjugants appeared to be unalterred from the original pJG22.

Total genomic DNA was isolated from Exo$^+$ and Exo$^-$ ANU2811(pJG22) transconjugants and analyzed in order to determine whether the nature of the recombination event that resulted in the formation of pJG22::Tn*5* plasmids was by double-reciprocal homologous recombination, gene duplication or by another means. Southern blots of electrophoresed *Eco*RI restricted genomic DNA was probed with ^{32}P-labelled pJG11 DNA (Fig. 4.3). The results indicated that both colony types had the 0.6 kb and the 0.6kb + Tn*5* *Eco*RI hybridizing fragments within their genomes. There were no novel hybridizing fragments; all bands could be accounted for as either belonging to the introduced plasmid or from the recipient genome. Since the plasmid DNA isolated from Exo$^+$ colonies appeared unalterred from pJG22 and the plasmids isolated from Exo$^-$ colonies now carried Tn*5* within the 0.6 kb *Eco*RI fragment with no detection of the original 0.6 kb *Eco*RI fragment on the plasmid pJG22::Tn*5*; it was concluded that the

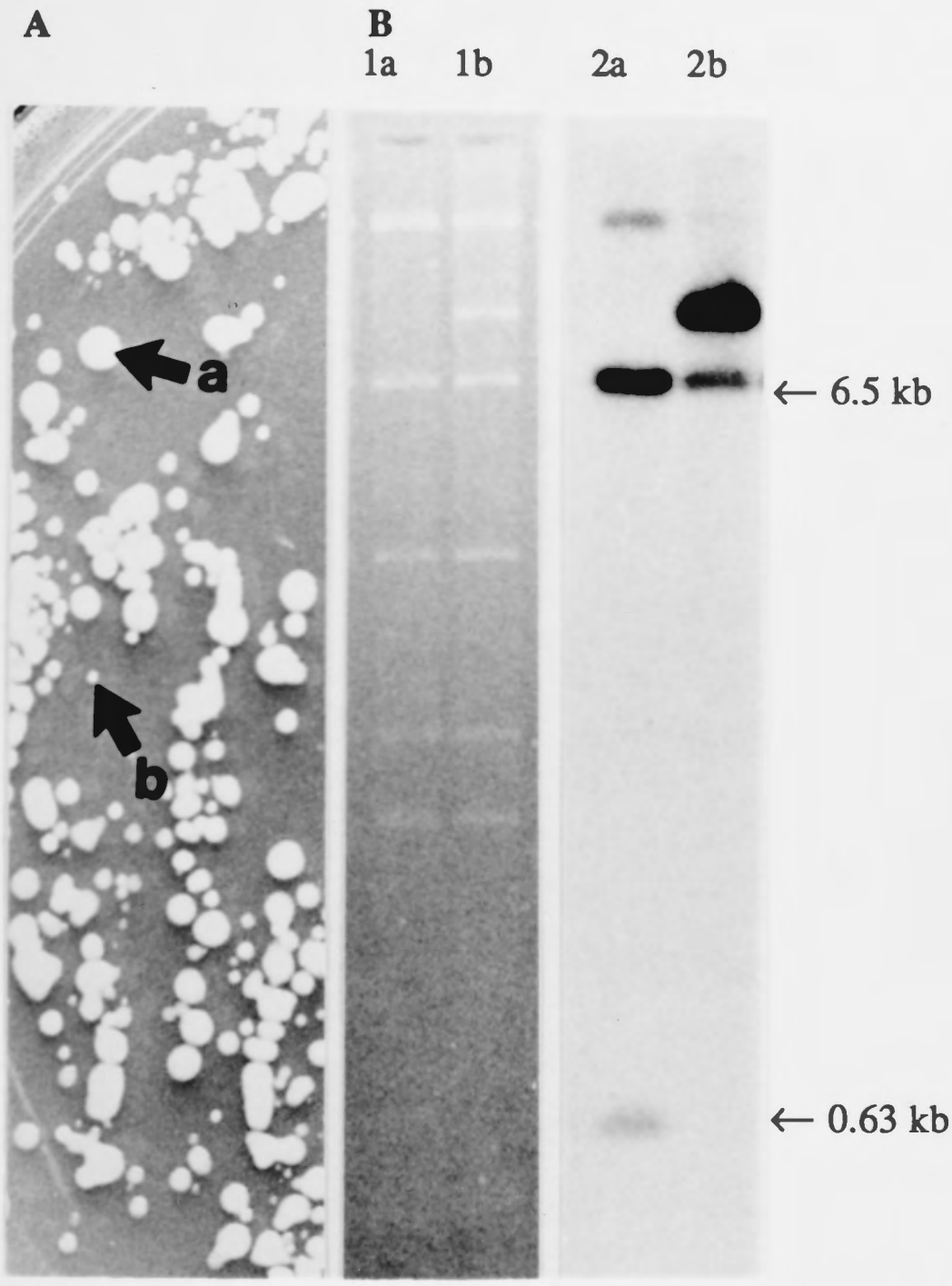


Fig. 4.2 Analysis of transconjugants resulting from the transfer of pJG22 into mutant strain ANU2811. **(A)** shows the Exo^+ (a) and Exo^- (b) colony types. **(B)** shows an analysis of plasmid DNA carried by Exo^+ and Exo^- transconjugants. Lanes: (1a), *Eco*RI restriction profile of the plasmid from an Exo^+ colony; (1b), *Eco*RI restriction profile of the plasmid from an Exo^- colony; (2a) and (2b), autoradiograph of a Southern blot of lanes 1a and 1b respectively, after hybridization with radioactively labeled plasmid pHC11. Plasmid pHC11 has a 6.5kb *Eco*RI insert, cloned from strain ANU2811, which carries the Tn*5* insertion. The 0.63kb band is the size of the wild-type *Eco*RI fragment and the 6.5kb band is this same *Eco*RI fragment carrying the Tn*5* insertion. The other hybridizing bands, common in both lanes 2a and 2b are due to cross hybridization between kanamycin resistance gene sequences located on the vector pJJ016 and present within Tn*5*.

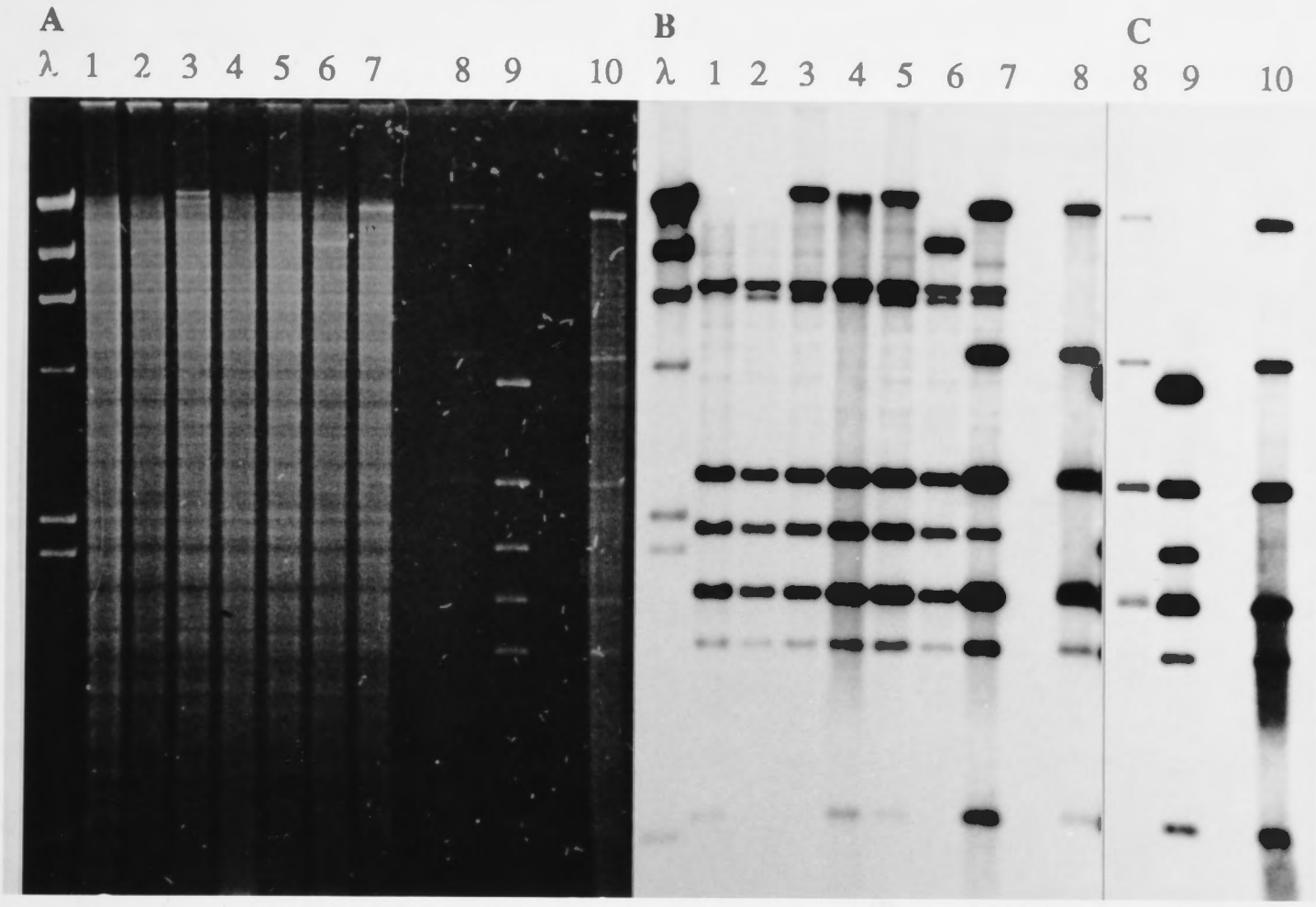


Fig. 4.3 Analysis of genomic DNA from transconjugants

(A) Electrophoresis of DNA through a 0.7% agarose gel.

Lane λ; *Hin*dIII restricted Lambda DNA,
Lane 1; *Eco*RI restricted ANU280 genomic DNA,
Lane 2; *Eco*RI restricted ANU2811 genomic DNA,
Lane 3; *Eco*RI restricted ANU2811(R'68.45) genomic DNA,
Lane 4; *Eco*RI restricted ANU2811(R'3222) genomic DNA,
Lane 5; *Eco*RI restricted ANU280(R'2811) genomic DNA,
Lane 6; *Eco*RI restricted ANU2811(pMP220) genomic DNA,
Lane 7; *Eco*RI restricted Exo^- ANU2811(pJG22) genomic DNA,
Lane 8; *Eco*RI restricted pJG22 plasmid DNA,
Lane 9; *Eco*RI restricted pJG11 plasmid DNA,
Lane 10; *Eco*RI restricted Exo^+ ANU2811(pJG22) genomic DNA.

Equal amounts of DNA were loaded into lanes 1 to 7 inclusive. The relative concentrations of DNA preparations were determined as accurately as possible by comparing U.V. absorbances and staining intensities with ethidium bromide. A small amount of pJG22 plasmid DNA was deliberately loaded into lane 8 to compensate for its increased molar ratio of target sequences. The non plasmid component of DNA in lane 10 had degraded.

(B) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled 10 kb *Bam*HI insert DNA from pJG11.

(C) Part of the same autoradiograph as in panel B, that was exposed for less time to more closely show the hybridization profiles.

plasmid pJG22::Tn*5* resulted from double-reciprocal recombination events between sequences flanking the Tn*5* insertion in the background genome and the homologous sequences carried on the introduced plasmid.

4.2.4 Genetic Analysis of Plasmids Recovered from Exo$^+$ and Exo$^-$ ANU2811(pJG22) Transconjugants

Double-reciprocal recombination events are normally very rare. Simon *et al.* (1983) reported the occurrence of such events over a 3.6 kb region of *nif* DNA in *R. meliloti* with a frequency of 2×10^{-5}. In ANU2811(pJG22) transconjugants, the recombination events were occurring at a comparable frequency of 1.7×10^{-4} (48% of the transconjugant frequency). The strong selection for these normally rare recombination events, combined with the observation that the transconjugation frequency was also reduced to 1% due to the cloned DNA, suggested that the wild-type allele was deleterious to cell growth when present at the copy number afforded by the IncP1 plasmid. In contradiction to this however, was the fact that 52% of the ANU2811(pJG22) transconjugants remained stably Exo$^+$. To ensure that plasmids from the Exo$^+$ transconjugants had not suffered a small undetectable deletion or rearrangement of only a few critical nucleotides, which rendered the putative deleterious gene(s) "harmless", plasmid DNA was recovered from Exo$^+$ and Exo$^-$ transconjugants and transformed into *E. coli*. These *E. coli* derivatives were used to retransfer these plasmids into the Exo$^-$ mutant strain ANU2811 as well as the original parental strain ANU280. The results for ANU2811 recipients were: (i), "unalterred" plasmids originally recovered from the Exo$^+$ transconjugants again produced a mixture of Exo$^+$ and Exo$^-$ colonies and (ii), pJG22::Tn*5* plasmids did not alter the Exo$^-$ phenotype of strain ANU2811 colonies. For strain ANU280 recipients the results were: (i), "unalterred" pJG22 conferred no phenotypic change upon colony morphology or growth and (ii), pJG22::Tn*5* conferred an Exo$^-$ phenotype upon 100% of the transconjugants. Furthermore, the "unalterred" pJG22 plasmids caused a 10^{-2} reduction in the frequency

of transconjugation in strain ANU2811 recipients, but not strain ANU280 recipients. Therefore, pJG22 plasmids recovered from the original Exo$^+$ ANU2811(pJG22) colonies were indeed unaltered. In contrast, pJG22::Tn*5* plasmids conferred a dominant Exo$^-$ phenotype when present in strain ANU280. The events proposed to occur upon transfer of pJG22 into strain ANU2811 are depicted in figure 4.4.

4.2.5 Copy-Numbers for pJG22 and R'3222 in *Rhizobium*

A high frequency of recombination events was not observed for strain ANU2811 transconjugants carrying the same wild-type *exo* DNA on the much larger (140 kb) R'3222 plasmid. The phenotype of such transconjugants was Exo$^+$ in virtually 100% of the cases, with the exception being rare occurrences of Exo$^-$ colonies arising at a frequency less than 10^{-3}. The frequency of conjugation with R'3222 was also very high. Therefore, R'3222 possessed none of the deleterious characteristics associated with the much smaller (30 kb) pJG22 plasmid. The possibility that the deleterious effects conferred by the wild-type DNA were dependent on its copy-number within the *Rhizobium* cells was investigated. Genomic DNAs (including chromosomes, megaplasmids and introduced plasmids) were isolated from: Exo$^+$ ANU280, Exo$^-$ ANU2811, Exo$^-$ ANU280(R'2811), Exo$^+$ ANU2811(R'3222), Exo$^+$ ANU2811(pJG22) and Exo$^-$ ANU2811(pJG22). A Southern blot of an agarose gel where equal amounts of *Eco*RI digested DNA from each strain had been electrophoresed, was probed with ^{32}P-labelled pJG11 DNA (Fig. 4.5). The intensity of hybridizing DNA bands was measured by using an LKB Bromma 2202 Ultroscan Laser Densitometer. The ratio of the intensities for hybridizing bands between the various genomic DNA preparations reflected their respective copy numbers. R'3222 was present in *Rhizobium* cells at approximately three copies per cell compared with approximately 10 copies per cell for pJG22.

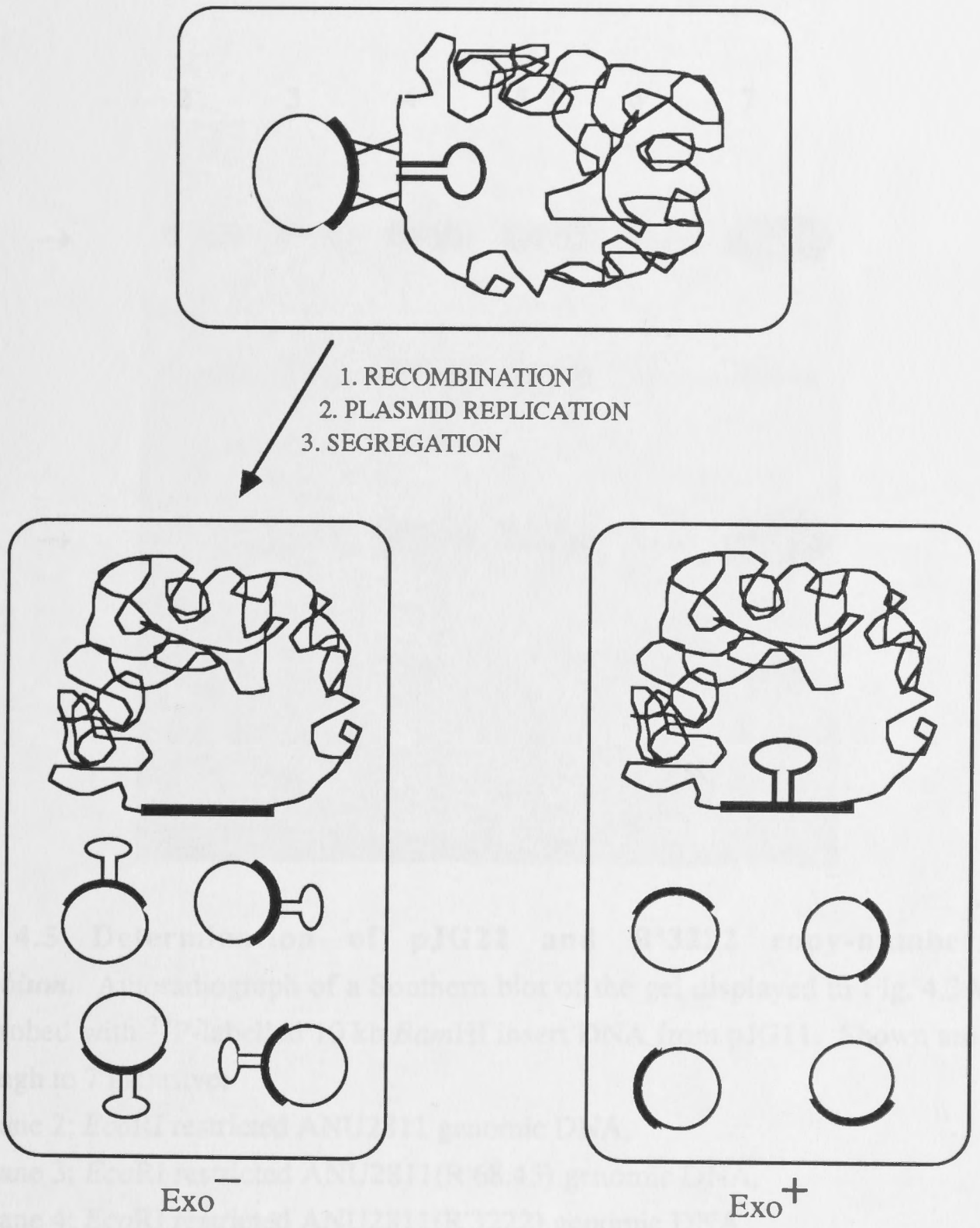


Fig. 4.4 Diagrammatic representation of events occurring in ANU2811(pJG22) transconjugant cells. In approximately 50% of the cells, double reciprocal recombination events take place between the *Rhizobium* DNA flanking the Tn*5* insertion and the homologous DNA present on the cloned insert of pJG22. The result is the exchange of alleles between the chromosome and the introduced plasmid. When such events occur the resultant phenotype of transconjugants remained Exo$^-$. When recombination events do not occur, the resultant phenotype becomes Exo$^+$ and appears to be true correction due to the introduced wild-type DNA.

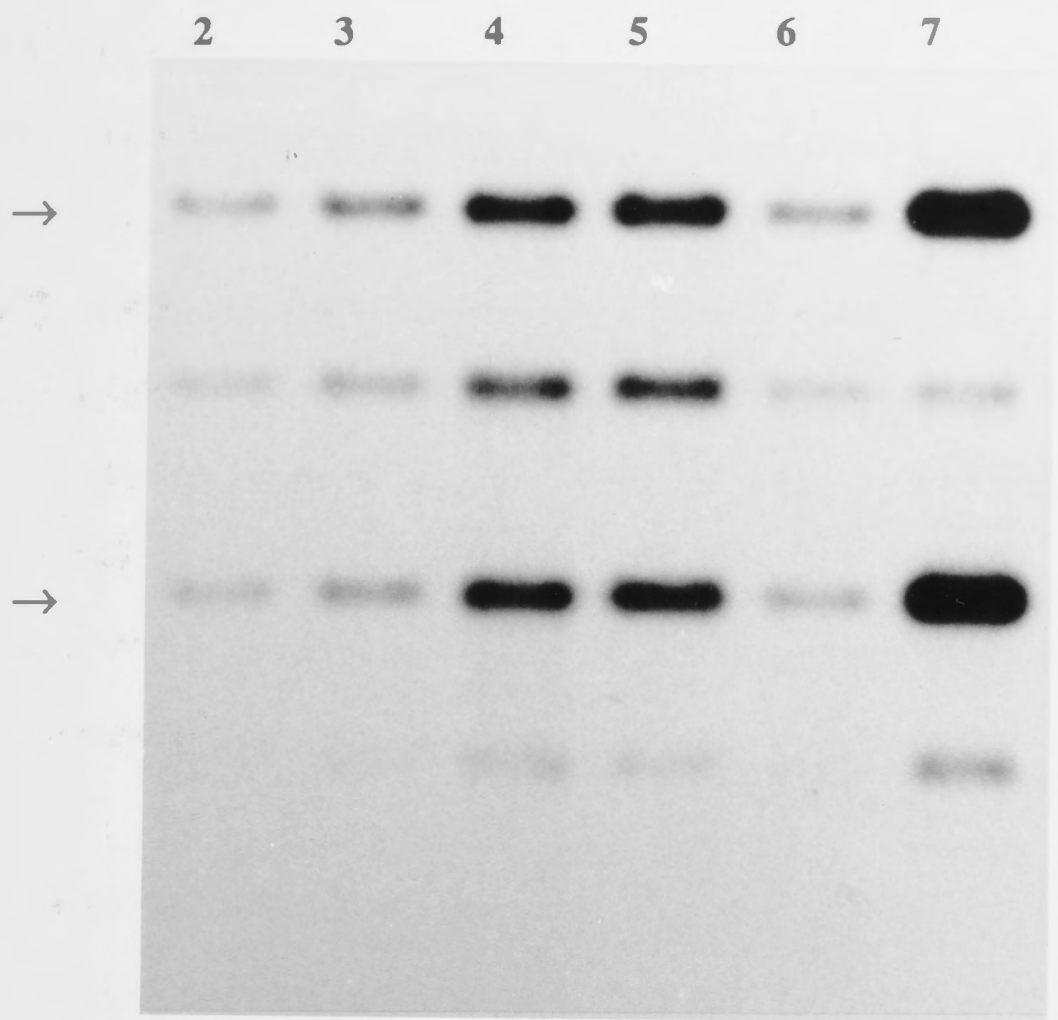


Fig. 4.5 Determination of pJG22 and R'3222 copy-numbers in *Rhizobium*. Autoradiograph of a Southern blot of the gel displayed in Fig. 4.3A, that was probed with ^{32}P-labelled 10 kb *Bam*HI insert DNA from pJG11. Shown are lanes 2 through to 7 inclusive:

Lane 2; *Eco*RI restricted ANU2811 genomic DNA,

Lane 3; *Eco*RI restricted ANU2811(R'68.45) genomic DNA,

Lane 4; *Eco*RI restricted ANU2811(R'3222) genomic DNA,

Lane 5; *Eco*RI restricted ANU280(R'2811) genomic DNA,

Lane 6; *Eco*RI restricted ANU2811(pMP220) genomic DNA,

Lane 7; *Eco*RI restricted Exo$^-$ ANU2811(pJG22) genomic DNA.

Equal amounts of genomic DNA were loaded into each of these lanes. Therefore the variations in intensities of hybridization to probe sequences reflects the relative copy numbers of these sequences in the respective transconjugant genomes. The bands indicated by arrows were used to measure the relative intensities of hybridization and hence, to quantify the copy-numbers of large R-prime plasmids and smaller IncP1 plasmids.

To further investigate copy-number effects, the 10 kb *Bam*HI fragment was cloned into the multi-copy IncQ vector pSUP106. Several unsuccessful attempts were made to transfer this recombinant plasmid (pJG100) into either strain ANU280 or strain ANU2811. The vector however, could be successfully transferred into either background with high efficiency. Plasmid pJG100 was mutagenized with the transposable element mini-Mu*dII*1734 (Castilho *et al.* 1984) for the purpose of inactivating the region causing the deleterious phenotype. Clones with insertions through out the 10 kb *Bam*HI fragment were isolated (Fig. 4.6). Attempts to transfer any of the mutated pJG100 plasmids into strains ANU280 or ANU2811 were either unsuccessful or achieved with very poor efficiency, suggesting that more than one locus was deleterious when present in very high doses within a cell.

4.2.6 Curing Transconjugants of Their Plasmids

In section 4.2.4, it was demonstrated that the plasmids recovered from Exo$^+$ ANU2811(pJG22) transconjugants were unalterred. The fact that 52% of the transconjugants could remain stably Exo$^+$ amidst strong selection (48%) for elimination of the wild-type allele carried by this plasmid, suggested the possibility that these Exo$^+$ transconjugants may have undergone some form of suppressor mutation at a site other than on the introduced plasmid. It was thought that such suppressor mutants could be isolated by curing the transconjugants of their IncP1 plasmid and upon the reintroduction of pJG22 into these suppressor mutants, perhaps 100% of the transconjugants would be Exo$^+$ and the frequency of transconjugants would be high.

Stable Exo$^+$ and Exo$^-$ ANU2811(pJG22) transconjugant isolates were grown in liquid cultures, from a small inoculum, in the absence of antibiotic selection, until late stationary phase. Dilutions of this culture were then plated out to single colonies on solid media without antibiotic selection for the plasmid. When the colonies were grown, they were then replica-plated onto solid media supplemented with tetracycline to select for those

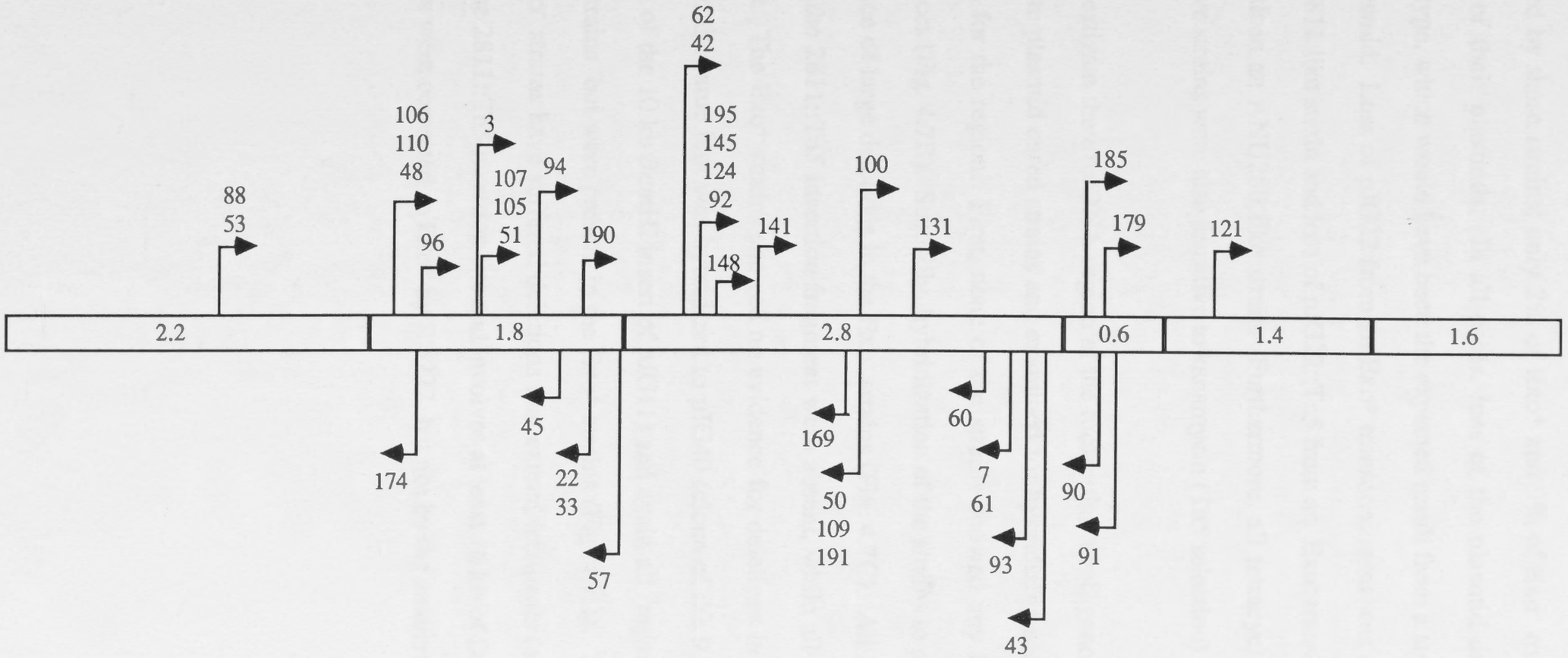


Fig. 4.6 *Eco*RI restriction map of of the 10 kb *Bam*HI insert of pJG100, showing insertions sites of mini-Mu*d*II*lac*. The DNA is represented by the box and the numbers within indicate the size in kb of the *Eco*RI restriction fragments. The vertical lines map the insertion sites of mini-Mu*d*II*lac* and the orientations are indicated with arrows in the directions of the fusions to *lacZ*. The numbers next to the arrows are reference numbers for the recombinant plasmids.

colonies still retaining the plasmid. It was observed that the plasmids were quite stably retained by these strains; only 2% of Exo$^+$ and 9% of Exo$^-$ transconjugants could be cured of their plasmids. In all cases, loss of the plasmid did not change the Exo phenotype, which would have been the expected result from a straight forward curing of the plasmid. Loss of pJG22 from an Exo$^+$ transconjugant was expected to result in an ANU2811 like strain and loss of pJG22::Tn*5* from an Exo$^-$ transconjugant was expected to result in an ANU280 like strain. Furthermore, all tetracycline (plasmid selection) sensitive strains were also sensitive to kanamycin (Tn*5* selection).

To investigate the *exo* DNA region at the molecular level, genomic DNA was isolated from the plasmid cured strains and examined for hybridizing sequences to ^{32}P-labelled probes for the region. First, none of the strains showed any hybridization to vector sequences (Fig. 4.7B). Secondly, hybridization of the strains to pJG11 demonstrated the existence of large deletions in the Exo$^-$ strains (Fig. 4.7C). All *Eco*RI fragments to the left of the 2811::Tn*5* insertion fragment were absent, while all those to the right were present. The Exo$^+$ strains showed no evidence for deletions in this region (Fig 4.7C). Finally, the same blot was hybridized to pJG40 (clone of the 9 kb *Bam*HI fragment to the left of the 10 kb *Bam*HI insert of pJG11) and again all fragments were absent in the Exo$^-$ strains, but were present in the Exo$^+$ strains (Fig. 4.7D). The result suggests that the Exo$^-$ strains have suffered deletions that extend leftwards (as portrayed in Fig. 3.2) from the 2811::Tn*5* insertion site and involves at least 16 kb of DNA. The Exo$^-$ deletion mutants were corrected to Exo$^+$ by R'3222, but not by the smaller clone of pJG22.

Fig. 4.7 Analysis of *exo* DNA from IncP1 plasmid cured transconjugants.

(A) Electrophoresis of DNA through a 0.7% agarose gel.

Lanes 1 to 4; *Eco*RI restricted genomic DNA isolated from IncP1 plasmid cured Exo$^-$ transconjugants,

Lane 5; *Eco*RI restricted genomic DNA isolated from ANU2811,

Lane 6; *Eco*RI restricted genomic DNA isolated from ANU280,

Lanes 7 to 10; *Eco*RI restricted genomic DNA isolated from IncP1 plasmid cured Exo$^+$ transconjugants,

Lane λ; *Hin*dIII restricted Lambda DNA,

Lane 11; *Eco*RI restricted pJG22 plasmid DNA.

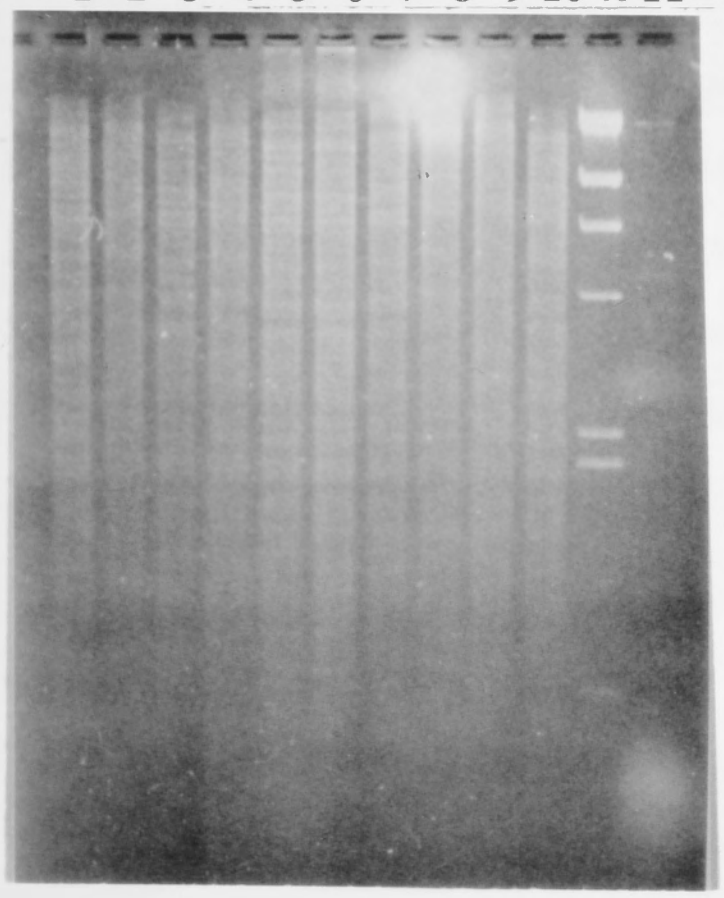
Equal amounts of DNA were not necessarily loaded into each well and only a very small quantity of pJG22 DNA was electrophoresed through lane 11 (not detected by ethidium bromide staining), to compensate for the increased concentration of target sequences.

(B) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled pMP220 vector DNA.

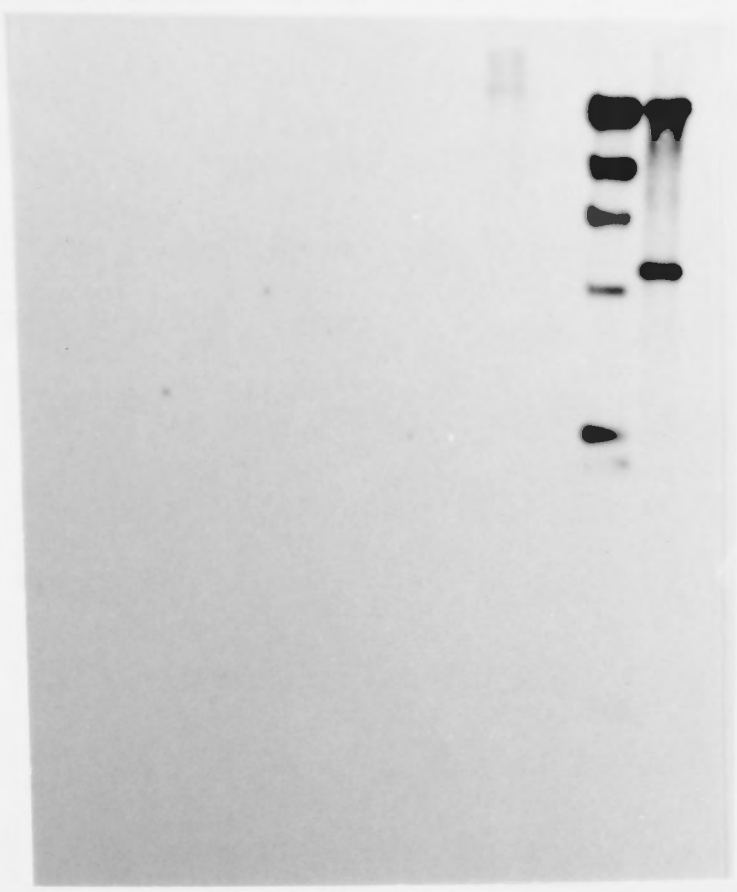
(C) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled 10 kb *Bam*HI insert DNA from pJG11.

(D) Autoradiograph of a Southern blot of the gel displayed in panel A, that was probed with ^{32}P-labelled 9 kb *Bam*HI insert DNA from pJG40.

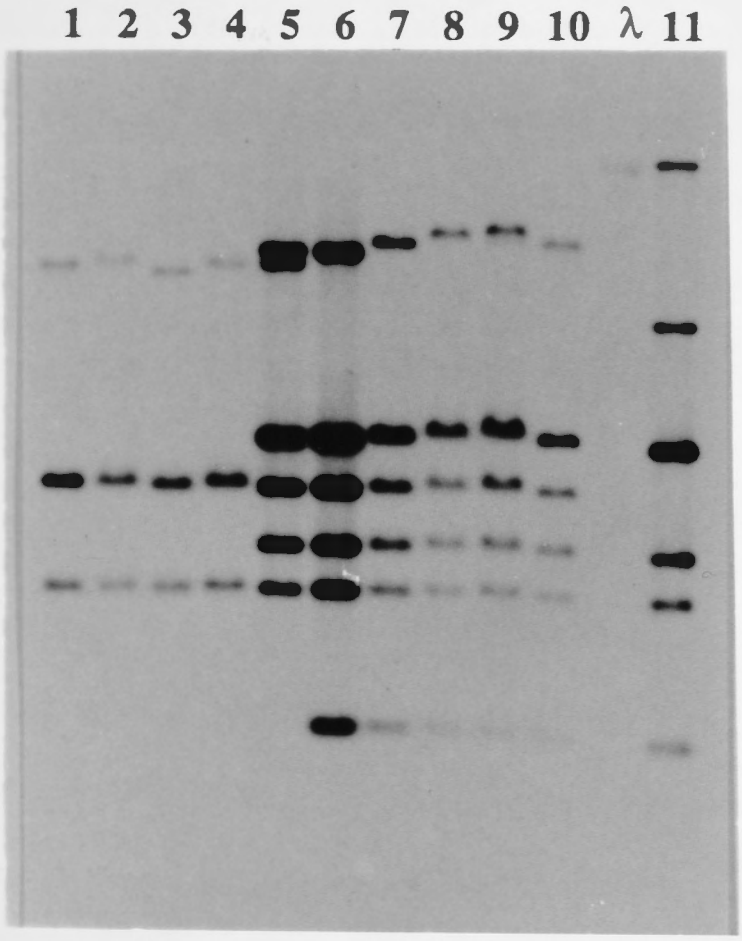
A


1 2 3 4 5 6 7 8 9 10 λ 11

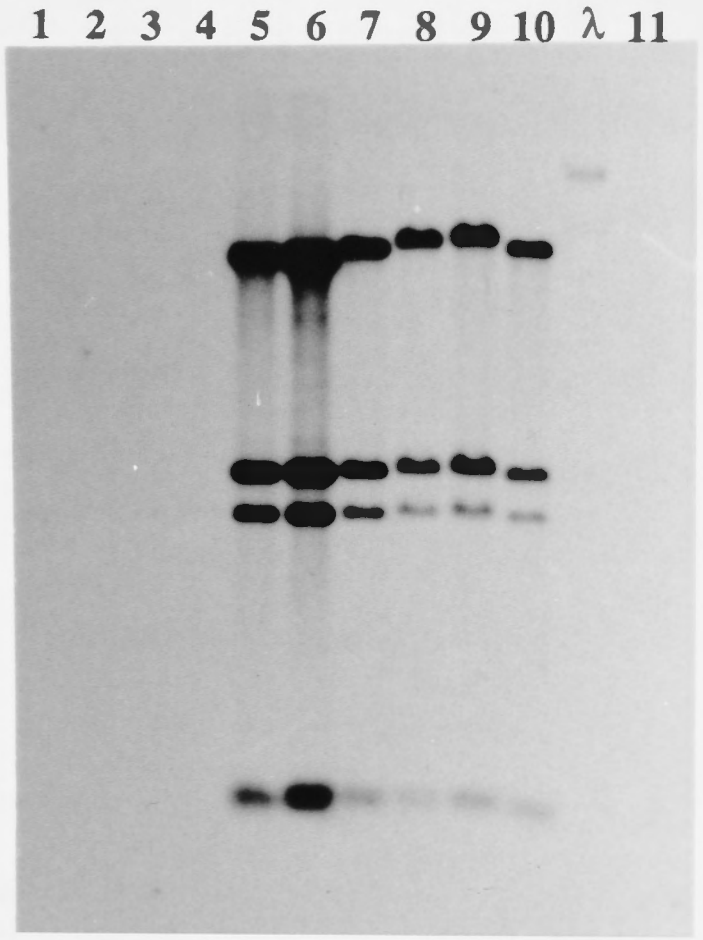
B


1 2 3 4 5 6 7 8 9 10 λ 11

C


1 2 3 4 5 6 7 8 9 10 λ 11

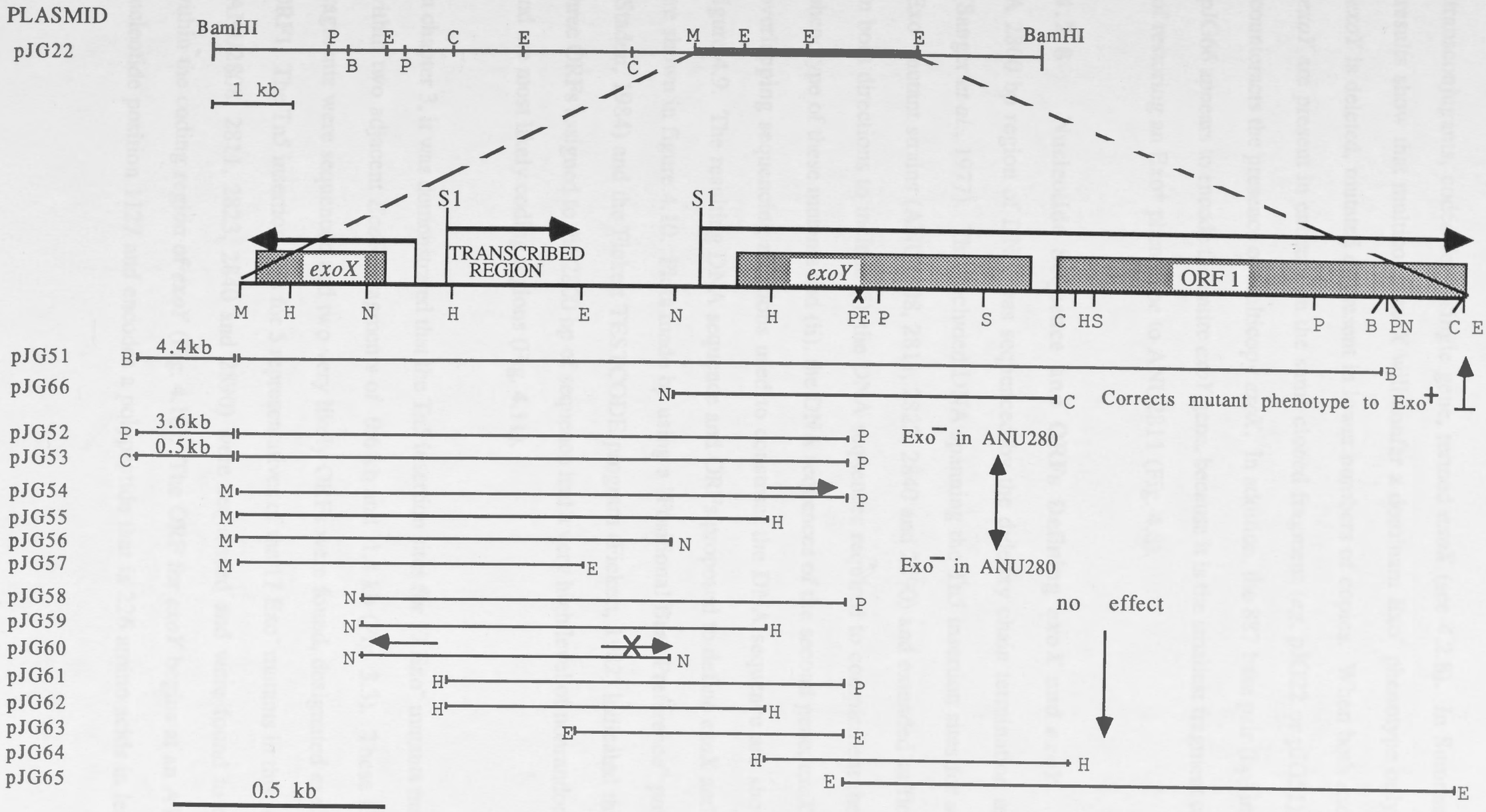
D


1 2 3 4 5 6 7 8 9 10 λ 11

4.2.7 Complementation Phenotype Associated with Subclones of the 10 kb *Bam*HI Fragment

As reported in section 4.2.4, the introduction of pJG22::Tn*5* into strain ANU280 resulted in the transconjugants being Exo$^-$. To determine whether the Exo$^-$ phenotype of ANU280(pJG22::Tn*5*) transconjugants was due to a dominant negative mutation or the presence of another element on the 10kb *Bam*HI insert of pJG22, a series of subclones from this *Bam*HI fragment were cloned into the IncP1 vector pMP220. Figure 4.8 is a summary of the fragments subcloned and their phenotypes, when present in the ANU280 or ANU2811 backgrounds. The subcloned fragments that are contained within the expanded portion of the map in figure 4.8 were constructed using insert DNA from sequencing subclones. Those fragments, which appear to be generated by partial restriction endonuclease digestion, were instead constructed by ligating the relevant fully digested fragments (agarose gel purified, section 2.5.4) and cloning the combined fragment into one of the Bluescript (Stratagene, San Diego, USA) sequencing vectors. To ensure that the original continuity of the DNA was preserved, the junctions of the ligated fragments were sequenced. These reconstructed partial fragments were then cloned into the vector pMP220 as single fragments, utilizing the wide choice of unique, flanking restriction sites present in the Bluescript polylinker. The choice of DNA fragments to reconstruct and clone, were best made with the knowledge gained from DNA sequencing of the region. The Tn*5* insertion in strain ANU2811 was found to occur in *exoY* (see 4.2.8). Restriction sites were chosen such that *exoY* was restricted at several sites within and proximal to the coding region and the fragments extended out in both directions for varying lengths. The results (Fig. 4.8) showed that the dominant Exo$^-$ phenotype associated with the plasmid borne locus could be attributed to a region of DNA that was 1kb upstream from *exoY*. As some of the subclones did not possess any of the *exoY* gene, the presence of truncated *exoY* gene product in strain ANU280 transconjugants was not responsible for the dominant Exo$^-$ phenotype. The region of DNA responsible for the generation of a dominant Exo$^-$ phenotype in ANU280

Fig. 4.8 Physical map of the 10kb *Bam*HI fragment involved in EPS synthesis. The nucleotide sequence has been determined for the expanded region of the map. The extremities of the subcloned fragments and the phenotypes associated with these fragments, when used in complementation experiments, are also shown. Plasmids pJG51 and pJG66 complement the Exo$^-$ mutant strain ANU2811 to Exo$^+$. Plasmids pJG52 through to pJG57 confer an Exo$^-$ phenotype when present in strain ANU280. Plasmids pJG58 through to pJG65 do not alter the phenotypes of either strains ANU280 or ANU2811. Symbols "→" and "↛" indicate presence or absence (respectively) of β-galactosidase activity associated with these fragments when fused to *lacZ* in the direction of the arrow. On the expanded map, shaded rectangles denote the putative coding regions of the sequenced genes and the arrows denote the direction of transcription. The sites of transcription initiation, as determined by S1 promoter mapping, are indicated by "S1". The abbreviated restriction sites indicated are: B, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hind*III; M, *Mlu*I; N, *Nru*I; P, *Pst*I; S, *Sma*I.


PLASMID
pJG22
BamHI
P
E
C
E
M
E
E
E
BamHI
B
P
C
1 kb
S1
S1
TRANSCRIBED
REGION
exoX
exoY
ORF 1
M H N H E N H PE P S C HS P B PN C E
pJG51
4.4kb
B
B
pJG66
N
C
Corrects mutant phenotype to Exo+
pJG52
3.6kb
P
P
pJG53
0.5kb
C
P
pJG54
M
P
pJG55
M
H
pJG56
M
N
pJG57
M
E
Exo− in ANU280
Exo− in ANU280
pJG58
N
P
pJG59
N
H
pJG60
N
N
pJG61
H
P
pJG62
H
H
pJG63
E
E
pJG64
H
H
pJG65
E
E
no effect
0.5 kb

transconjugants, coded for a single gene, termed *exoX* (see 4.2.8). In Summary, the results show that multicopy *exoX* will confer a dominant Exo$^-$ phenotype only when *exoY* is deleted, mutated or present in lower numbers of copies. When both *exoX* and *exoY* are present in entirety on the same cloned fragment (*eg*. pJG22 or pJG51), *exoY* counteracts the presence of multicopy *exoX*. In addition, the 887 base pair (bp) insert of pJG66 appears to encode the entire *exoY* gene, because it is the smallest fragment capable of restoring an Exo$^+$ phenotype to ANU2811 (Fig. 4.8).

4.2.8 Nucleotide Sequence and ORFs Defining *exoX* and *exoY*

A 2800 bp region of DNA was sequenced by the dideoxy chain termination method (Sanger *et al.*, 1977). This included DNA spanning the Tn*5* insertion sites for several Exo$^-$ mutant strains (ANU2808, 2811, 2823, 2840 and 2890) and extended sufficiently in both directions to include (i), the DNA sequences required to complement the Exo$^-$ phenotype of these mutants and (ii), the DNA sequences of the second gene, *exoX*. The overlapping sequencing reactions used to construct the DNA sequence are shown in figure 4.9. The resulting DNA sequence and ORFs proposed to define *exoX* and *exoY* are shown in figure 4.10. Plots made by using a "Positional Base Preference" program (Staden, 1984) and the Fickett TESTCODE program (Fickett, 1982) indicated that the three ORFs assigned to the 2800 bp of sequence had a very high level of nonrandomness and are most likely coding regions (Fig. 4.11).

In chapter 3, it was demonstrated that the Tn*5* insertion sites for 17 Exo$^-$ mutants mapped within two adjacent *Eco*RI fragments of 0.6 kb and 1.4 kb (Fig. 3.3). These *Eco*RI fragments were sequenced and two very likely ORFs were found, designated *exoY* and ORF1. The Tn*5* insertion sites for 5 representatives of the 17 Exo$^-$ mutants in this region (ANU2808, 2811, 2823, 2840 and 2890) were sequenced and were found to occur within the coding region of *exoY* (Fig. 4.10). The ORF for *exoY* begins at an ATG at nucleotide position 1127 and encodes a polypeptide that is 226 amino acids in length.

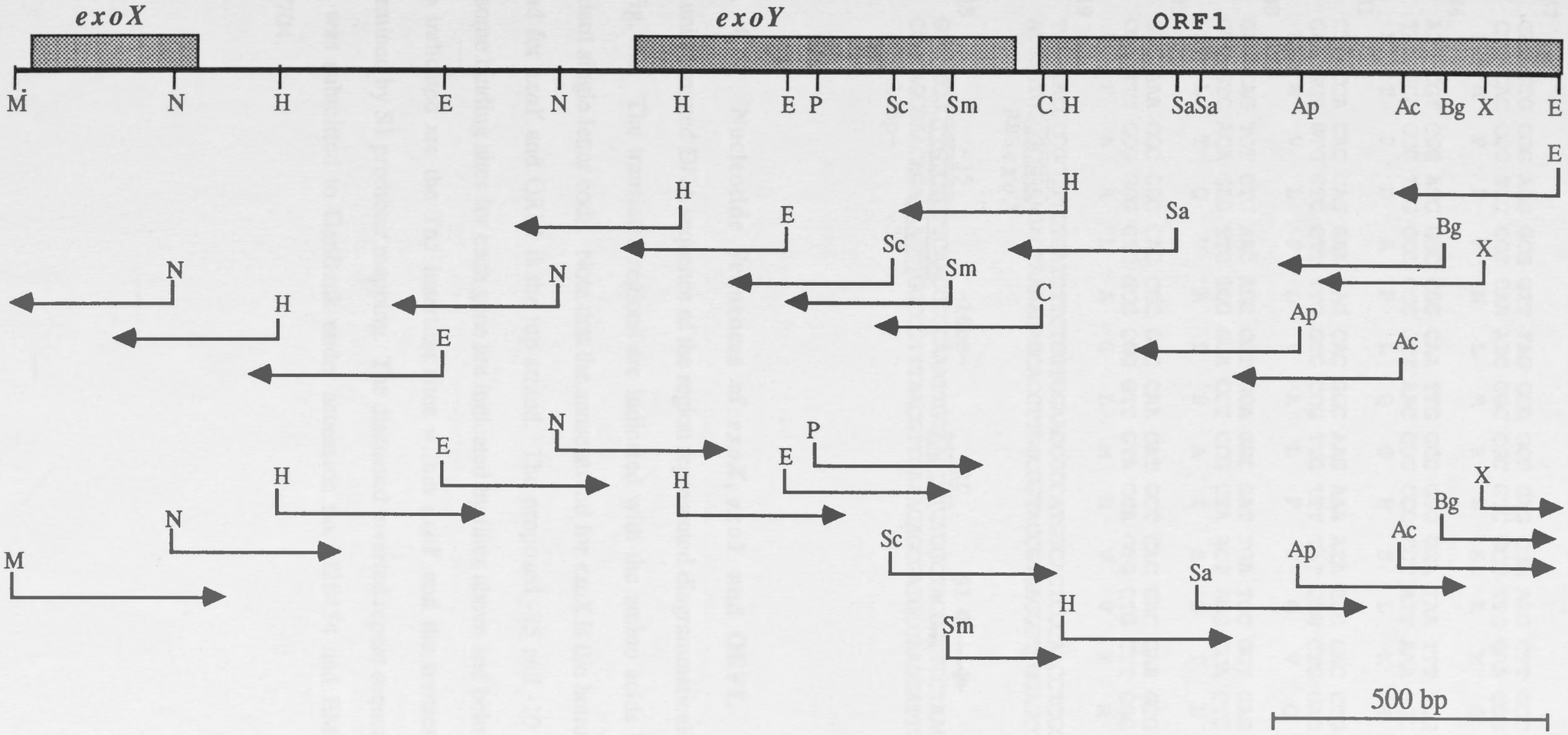


Fig. 4.9 Sequencing strategy for the *exo* region.

A partial restriction map of the sequenced region (presented in Fig. 4.8 and 4.10), showing only those restriction sites that were used for cloning. The arrows indicate the lengths and directions of the sequence obtained at each site. Restriction sites are abbreviated as follows: Ac, *Acc*I; Ap, *Apa*I; Bg, *Bgl*II; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nru*I; P, *Pst*I; Sa, *Sau*3AI; Sc, *Sac*I; Sm, *Sma*I; X, *Xho*I.

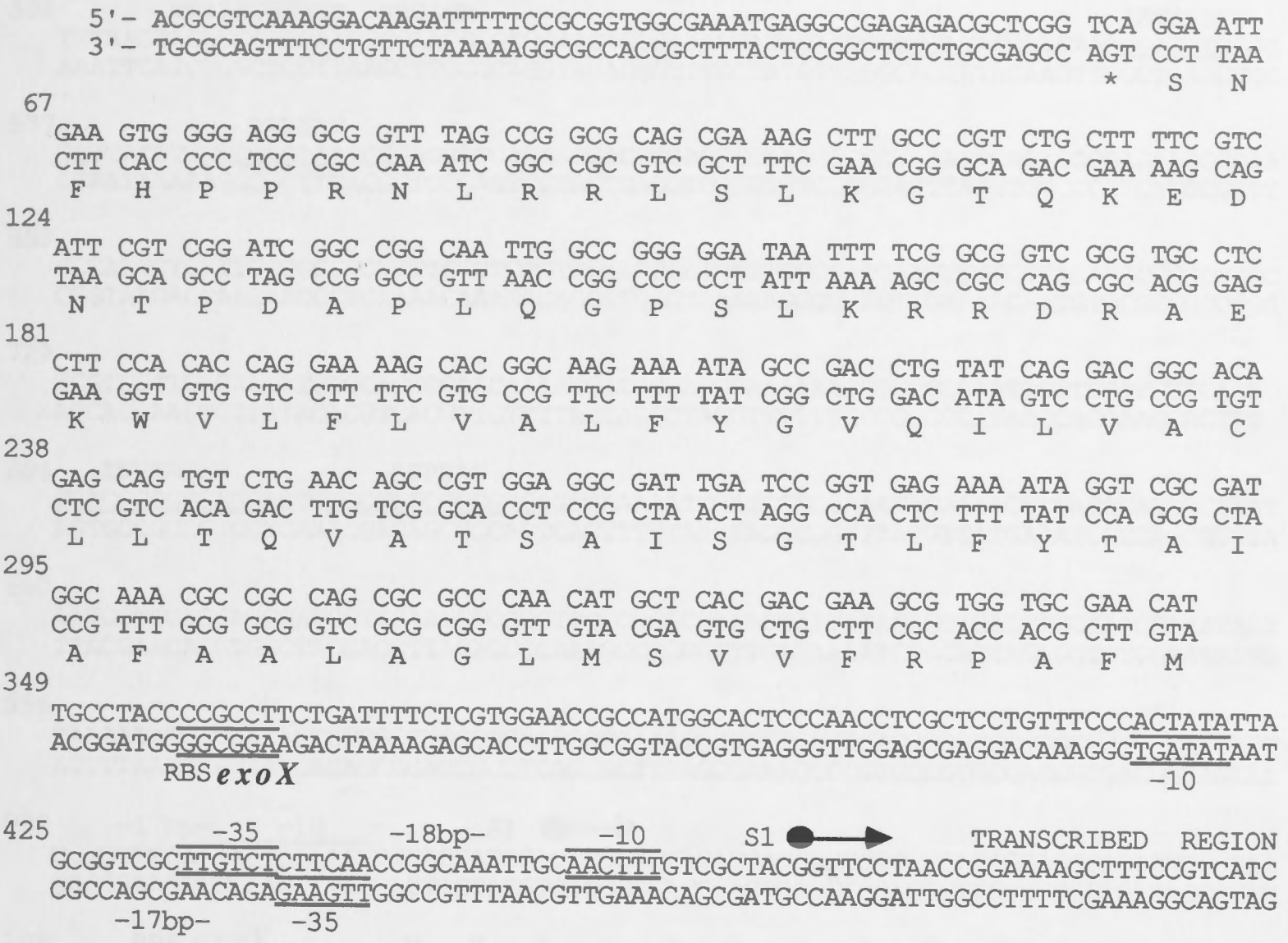


Fig. 4.10 Nucleotide Sequences of *exoX*, *exoY* and ORF1.

The uninterrupted DNA sequence of the region represented diagrammatically in the expanded portion of Fig. 4.8. The translated regions are indicated with the amino acids being represented by the standard single letter code. Note that the sense strand for *exoX* is the bottom strand, while the sense strand for *exoY* and ORF1 is the top strand. The proposed -35 and -10 transcription signals and ribosome binding sites for each gene are indicated by lines above and below the relevant sequences. Also indicated are the Tn*5* insertion sites within *exoY* and the transcription initiation sites as determined by S1 promoter mapping. The discussed inverted repeat sequences are underlined. This data was submitted to GenBank under accession no. M28454 and EMBL under accession no. X16704.

```
501        TRANSCRIBED  REGION                                          INVERTED
   TTTAAGTAGACCGAGCAATTCGAACGGGTCCATCTGCCAAACGATACAATCCCTGATATGTTCAACGGATTTGGGG
   AAATTCATCTGGCTCGTTAAGCTTGCCCAGGTAGACGGTTTGCTATGTTAGGGACTATACAAGTTGCCTAAACCCC

577              REPEAT
   GATTATTTTTCCGCGAAATCCGAGGGTCAAGATGACTCGAGCCCAAAGCCCCTAAATTCACAGGGGAGAACGCGAA
   CTAATAAAAAGGCGCTTTAGGCTCCCAGTTCTACTGAGCTCGGGTTTCGGGGATTTAAGTGTCCCCTCTTGCGCTT

653
   GCCATTCTGATTGTTGGGACTTTTGTTTCTGTCGAAGCGATTTTTCCCGATCAACTTTTGTTACAGGCGCCCGGCC
   CGGTAAGACTAACAACCCTGAAAACAAAGACAGCTTCGCTAAAAAGGGCTAGTTGAAAACAATGTCCGCGGGCCGG

729
   TCGTGCTTGAGAATATGTGCAGTGCAACAAAATGATGCGATGCAAAAAACGGCGGCGAATTCGTCGTTCGTCAAA
   AGCACGAACTCTTATACACGTCACGTTGTTTTACTACGCTACGTTTTTTGCCGCCGCTTAAGCAGCAAGCAGTTT

804   INVERTED            REPEAT
   TCACGGCGGCAGCAGTTTGCTGTCGCGGTGAGTGAAAAATTGATCCGCCAAATACAATACTTTAGCCATCGCAAAT
   AGTGCCGCCGTCGTCAAACGACAGCGCCACTCACTTTTTAACTAGGCGGTTTATGTTATGAAATCGGTAGCGTTTA

880
   AATGCTTGTGCACCGATGCTGGAATGTCGCGTTTCCGGGCCAAACTATTTTAGACGTGATCTCCAAACCATATACT
   TTACGAACACGTGGCTACGACCTTACAGCGCAAAGGCCCGGTTTGATAAAATCTGCACTAGAGGTTTGGTATATGA

956                                                                  -35
   TAAAATTTGGTAATAGTGTCGCCTGGCGCAGTCGCGAAATCGCTTTGGGGCACGGCGCGCTGGCGCTGCCATCATT
   ATTTTAAACCATTATCACAGCGGACCGCGTCAGCGCTTTAGCGAAACCCCGTGCCGCGCGACCGCGACGGTAGTAA

1032   -17bp-    -10          S1
   CCGCCTTCAAAAATCAAATTCTCACCATACACTCGCGCTAGATGACGCCCCGTCCCACGGGACCGACAATCGCCAA
   GGCGGAAGTTTTTAGTTTAAGAGTGGTATGTGAGCGCGATCTACTGCGGGGCAGGGTGCCCTGGCTGTTAGCGGTT

1108     RBS exoY          M   K   S   A   T   R   S   A   T   T   A   F   F   I
   CGTAAATGGAGTCACCTCT  ATG AAG TCC GCG ACT CGC TCG GCC ACT ACG GCT TTT TTT ATT
   GCATTTACCTCAGTGGAGA  TAC TTC AGG CGC TGA GCG AGC CGG TGA TGC CGA AAA AAA TAA

                    Tn5::2811
1169 P   Q   E   T   G   A   I   R   P   I   G   G   I   S   K   R   S   F   D
   CCG CAG GAG ACC GGA GCA ATC CGG CCG ATC GGC GGA ATC TCC AAG CGA AGC TTC GAC
   GGC GTC CTC TGG CCT CGT TAG GCC GGC TAG CCG CCT TAG AGG TTC GCT TCG AAG CTG

1226 V   L   I   A   I   L   A   L   I   A   L   S   P   L   F   L   L   V   M
   GTT CTC ATT GCC ATC CTG GCA CTT ATC GCC CTC AGT CCG CTT TTC CTG CTC GTC ATG
   CAA GAG TAA CGG TAG GAC CGT GAA TAG CGG GAG TCA GGC GAA AAG GAC GAG CAG TAC

1283 G   L   V   K   F   S   D   G   G   S   I   F   Y   G   H   R   R   I   G
   GGG CTG GTT AAG TTC TCG GAC GGC GGC AGC ATT TTC TAC GGC CAT CGC CGA ATC GGC
   CCC GAC CAA TTC AAG AGC CTG CCG CCG TCG TAA AAG ATG CCG GTA GCG GCT TAG CCG

1340 H   N   G   Q   T   F   K   C   L   K   F   R   T   M   M   E   N   G   D
   CAT AAC GGC CAG ACC TTC AAG TGC CTC AAG TTC CGC ACG ATG ATG GAA AAC GGC GAT
   GTA TTG CCG GTC TGG AAG TTC ACG GAG TTC AAG GCG TGC TAC TAC CTT TTG CCG CTA

1397 R   V   L   Q   E   F   F   K   S   N   P   A   A   Y   E   E   W   R   T
   CGG GTC CTG CAG GAA TTC TTC AAG TCG AAT CCT GCC GCC TAC GAG GAA TGG CGT ACG
   GCC CAG GAC GTC CTT AAG AAG TTC AGC TTA GGA CGG CGG ATG CTC CTT ACC GCA TGC
```

Fig. 4.10 Continued

```
                                                                  Tn5::2823/90
1454 T   R   K   L   Q   D   D   P   R   V   T   V   V   G   S   V   L   R   K
     ACC CGC AAG CTG CAG GAC GAT CCG CGC GTC ACC GTG GTC GGA AGC GTC CTT CGC AAG
     TGG GCG TTC GAC GTC CTG CTA GGC GCG CAG TGG CAC CAG CCT TCG CAG GAA GCG TTC

1511 L   S   L   D   E   L   P   Q   L   L   N   I   I   R   G   E   M   S   I
     CTC AGC CTC GAT GAA CTG CCC CAG CTC CTC AAC ATC ATT CGT GGT GAG ATG AGC ATC
     GAG TCG GAG CTA CTT GAC GGG GTC GAG GAG TTG TAG TAA GCA CCA CTC TAC TCG TAG

                                                  Tn5::2808
1568 V   G   P   R   P   V   V   E   D   E   L   E   L   Y   D   S   A   A   E
     GTC GGC CCG CGC CCG GTG GTC GAA GAT GAA CTG GAG CTC TAC GAT TCG GCC GCG GAG
     CAG CCG GGC GCG GGC CAC CAG CTT CTA CTT GAC CTC GAG ATG CTA AGC CGG CGC CTC

1625 F   Y   L   R   S   R   P   G   L   T   G   L   W   Q   I   S   G   R   N
     TTC TAT CTG CGC TCG CGT CCC GGC CTG ACC GGC CTC TGG CAG ATC AGC GGC CGC AAC
     AAG ATA GAC GCG AGC GCA GGG CCG GAC TGG CCG GAG ACC GTC TAG TCG CCG GCG TTG

                                                              Tn5::2840
1682 D   V   S   Y   A   T   R   V   A   F   D   T   H   Y   V   Q   N   W   S
     GAT GTG TCC TAT GCC ACC CGG GTG GCC TTC GAT ACG CAC TAT GTC CAG AAC TGG TCG
     CTA CAC AGG ATA CGG TGG GCC CAC CGG AAG CTA TGC GTG ATA CAG GTC TTG ACC AGC

1739 L   L   A   D   L   V   I   V   F   K   T   I   P   A   V   C   L   S   R
     CTC CTT GCC GAC CTC GTC ATC GTC TTC AAG ACG ATC CCC GCC GTC TGC CTC TCC CGC
     GAG GAA CGG CTG GAG CAG TAG CAG AAG TTC TGC TAG GGG CGG CAG ACG GAG AGG GCG

1796 G   S   Y   *                                                       M
                                                          RBS ORF1
     GGC AGC TAC TGA AATCTTGGCGTCTGCTCGCGGTAGAGCAAGACGTCAAGCAACGGGAAAAATCCCAA ATG
     CCG TCG ATG ACT TTAGAACCGCAGACGAGCGCCATCTCGTTCTGCAGTTCGTTGCCCTTTTAGGGTT TAC

1866 Q   S   I   R   V   L   G   A   P   A   G   S   R   K   L   L   H   F   A
     CAA TCG ATA AGA GTT CTA GGG GCG CCT GCC GGC TCC CGC AAG CTT CTC CAT TTC GCC
     GTT AGC TAT TCT CAA GAT CCC CGC GGA CGG CCG AGG GCG TTC GAA GAG GTA AAG CGG

1923 R   L   A   L   C   A   A   L   V   V   S   G   A   A   V   A   R   A   D
     CGC CTC GCA CTC TGC GCG GCG CTT GTC GTC TCC GGT GCC GCC GTT GCC CGG GCG GAC
     GCG GAG CGT GAG ACG CGC CGC GAA CAG CAG AGG CCA CGG CGG CAA CGG GCC CGC CTG

1980 D   Y   R   L   G   V   M   D   K   L   R   V   R   V   A   E   W   Q   T
     GAC TAT CGG CTC GGC GTT ATG GAC AAA TTG CGG GTT CGC GTT GCC GAA TGG CAG ACC
     CTG ATA GCC GAG CCG CAA TAC CTG TTT AAC GCC CAA GCG CAA CGG CTT ACC GTC TGG

2037 A   E   G   A   V   R   D   W   S   A   V   S   G   E   Y   T   V   G   A
     GCC GAG GGC GCG GTC CGG GAT TGG TCG GCC GTC AGC GGC GAG TAC ACG GTC GGA GCA
     CGG CTC CCG CGC CAG GCC CTA ACC AGC CGG CAG TCG CCG CTC ATG TGC CAG CCT CGT

2094 S   G   S   V   S   L   P   F   V   G   D   L   P   A   S   G   R   T   T
     TCG GGC AGC GTG TCG CTG CCT TTC GTA GGT GAT CTG CCC GCC TCG GGC CGG ACG ACG
     AGC CCG TCG CAC AGC GAC GGA AAG CAT CCA CTA GAC GGG CGG AGC CCG GCC TGC TGC

2151 T   E   V   A   E   E   I   G   I   K   M   Q   K   L   F   G   L   R   D
     ACG GAA GTC GCC GAA GAG ATC GGC ATC AAG ATG CAG AAG CTG TTC GGT CTG CGC GAC
     TGC CTT CAG CGG CTT CTC TAG CCG TAG TTC TAC GTC TTC GAC AAG CCA GAC GCG CTG
```

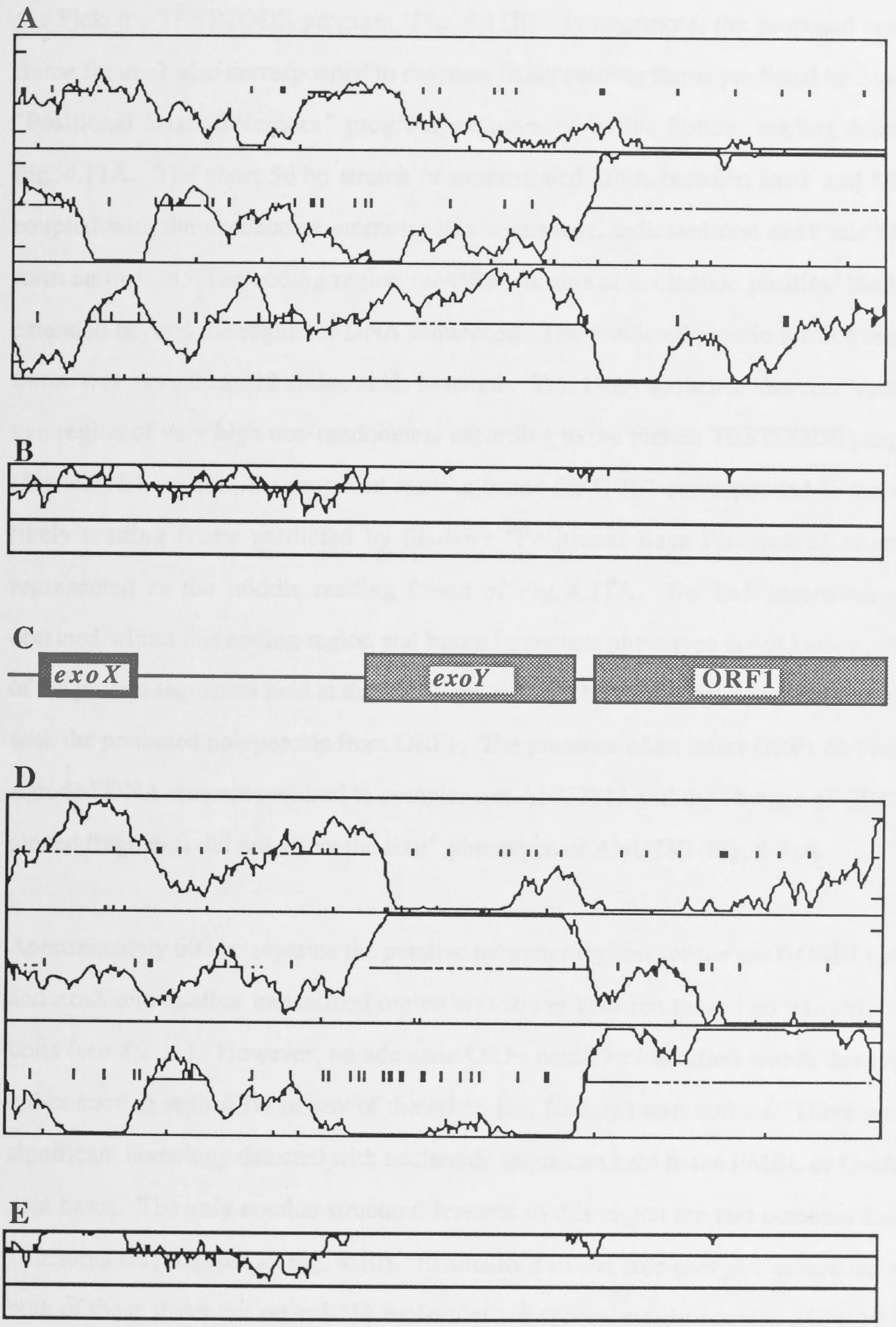
Fig. 4.10 Continued

```
2208 R   P   S   A   S   V   E   M   A   Q   Y   R   P   V   Y   L   Y   G   E
     CGG CCG TCT GCC TCG GTC GAA ATG GCA CAA TAC CGG CCG GTC TAT CTC TAT GGC GAG
     GCC GGC AGA CGG AGC CAG CTT TAC CGT GTT ATG GCC GGC CAG ATA GAG ATA CCG CTC

2265 V   E   T   P   G   E   Y   P   Y   A   P   N   L   T   V   L   K   A   V
     GTG GAG ACA CCT GGC GAG TAC CCC TAC GCT CCC AAT CTG ACG GTC CTC AAG GCA GTC
     CAC CTC TGT GGA CCG CTC ATG GGG ATG CGA GGG TTA GAC TGC CAG GAG TTC CGT CAG

2322 S   L   S   G   G   L   R   R   G   P   T   G   Q   R   F   A   R   D   Y
     AGC CTC AGC GGC GGG CTG CGC CGG GGC CCG ACC GGC CAG CGC TTC GCC CGC GAC TAT
     TCG GAG TCG CCG CCC GAC GCG GCC CCG GGC TGG CCG GTC GCG AAG CGG GCG CTG ATA

2379 I   A   A   N   G   D   S   S   V   Q   V   A   E   R   N   R   L   L   I
     ATC GCC GCG AAT GGC GAT TCG TCC GTC CAG GTT GCC GAG CGC AAC CGG CTG CTC ATT
     TAG CGG CGC TTA CCG CTA AGC AGG CAG GTC CAA CGG CTC GCG TTG GCC GAC GAG TAA

2436 R   R   A   R   L   Q   A   E   I   A   K   H   D   K   I   E   L   P   E
     CGG CGT GCC CGT CTG CAG GCG GAG ATA GCC AAG CAT GAC AAG ATT GAG CTG CCC GAG
     GCC GCA CGG GCA GAC GTC CGC CTC TAT CGG TTC GTA CTG TTC TAA CTC GAC GGG CTC

2493 E   L   K   N   A   P   G   V   D   K   L   L   E   S   E   T   A   L   M
     GAA CTC AAG AAC GCC CCC GGC GTC GAC AAG TTG CTC GAA AGC GAG ACG GCG CTG ATG
     CTT GAG TTC TTG CGG GGG CCG CAG CTG TTC AAC GAG CTT TCG CTC TGC CGC GAC TAC

2550 V   S   R   D   K   R   Q   D   R   Q   L   D   A   L   A   D   L   K   S
     GTT TCG CGC GAC AAG CGG CAG GAC CGC CAG CTC GAC GCC CTG GCA GAT CTC AAG TCC
     CAA AGC GCG CTG TTC GCC GTC CTG GCG GTC GAG CTG CGG GAC CGT CTA GAG TTC AGG

2607 L   L   Q   S   E   I   E   S   L   A   K   K   A   E   T   Q   Q   R   Q
     CTG CTG CAG AGC GAG ATC GAA TCG CTC GCG AAG AAG GCC GAA ACG CAG CAG CGT CAG
     GAC GAC GTC TCG CTC TAG CTT AGC GAG CGC TTC TTC CGG CTT TGC GTC GTC GCA GTC

2664 L   E   L   A   M   E   D   R   D   K   V   D   S   L   A   E   K   G   L
     CTC GAG CTC GCC ATG GAG GAC CGC GAC AAG GTC GAC AGC CTC GCC GAG AAG GGC CTG
     GAG CTC GAG CGG TAC CTC CTG GCG CTG TTC CAG CTG TCG GAG CGG CTC TTC CCG GAC

2721 A   L   S   Q   R   K   L   S   L   E   Q   R   V   A   D   V   Q   S   Q
     GCG CTG AGC CAG CGC AAG CTC TCG CTT GAG CAG CGG GTT GCC GAC GTG CAG TCG CAG
     CGC GAC TCG GTC GCG TTC GAG AGC GAA CTC GTC GCC CAA CGG CTG CAC GTC AGC GTC

2778 L   L   D   I   D   T   N
     CTT CTC GAC ATC GAT ACG AAT TC -3'
     GAA GAG CTG TAG CTA TGC TTA AG -5'
```

Fig. 4.10 Continued

Fig. 4.11 Computer based predictions of coding regions.

(**A**) Analysis of the top DNA strand in figure 4.10 with the positional base preference program (Staden, 1984). Coding probabilities for the three reading frames are shown. The reading frame for ORF1 is in the middle and the reading frame of *exoY* is on the bottom. Stop codons are shown as short vertical bars at the 50% level and possible ATG start codons are shown as short vertical bars at the 0% level. (**B**) Analysis of the top DNA strand in figure 4.10 for non-randomness using Fickett's (1982) TESTCODE program. (**C**) A genetic map of the 2800 bp region. (**D**) Analysis of the bottom DNA strand in figure 4.10 with the positional base preference program (Staden, 1984). The reading frame for *exoX* is on the top. (**E**) Analysis of the bottom DNA strand in figure 4.10 for non-randomness using Fickett's (1982) TESTCODE program.


A
B
C
exoX
exoY
ORF1
D
E

This DNA sequence corresponded to a region of very high non-randomness according to the Fickett's TESTCODE program (Fig. 4.11B). Furthermore, the proposed reading frame for *exoY* also corresponded to the most likely reading frame predicted by Staden's "Positional Base Preference" program, represented as the bottom reading frame of Fig. 4.11A. The short 56 bp stretch of untranslated DNA between *exoY* and ORF1, coupled with the absence of promoter-like sequences, indicated that *exoY* and ORF1 form an operon. The coding region for ORF1 begins at nucleotide position 1863 and extended beyond the region of DNA sequenced. The predicted protein for this reading frame was more than 312 amino acids in length. This DNA sequence also corresponded to a region of very high non-randomness according to the Fickett TESTCODE program (Fig. 4.11B). Again, the proposed reading frame for ORF1 corresponded to the most likely reading frame predicted by Staden's "Positional Base Preference" program; represented as the middle reading frame of Fig. 4.11A. No Tn*5* insertions were obtained within this coding region and hence its mutant phenotype is not known. None of the protein sequences held in the NBRF protein data base had any detectable homology with the predicted polypeptide from ORF1. The presence of an intact ORF1 on plasmid encoded DNA was not required to complement ANU2811 and the absence of ORF1 on cloned fragments did not affect the Exo^+ phenotype of ANU280 (Fig. 4.8).

Approximately 600 bp separate the putative promoter regions for the *exoY*-ORF1 operon and *exoX* and another transcribed region was found between these two transcriptional units (see 4.2.11). However, no adequate ORFs could be identified within this region either starting with ATG or any of the other, less frequent start codons. There was no significant homology detected with nucleotide sequences held in the EMBL or GenBank data bases. The only notable structural features in this region are two potential hairpin structures (highlighted in Fig. 4.10). Estimations of the free energies associated with both of these structures on mRNA molecules are approximately equal at $\Delta G° = -19$ kcal mol^{-1} in 1M NaCl at 37°C (Freier *et al.*, 1986). In the entire 2800 bp of sequence, these

are the two best palindromic sequences and either, may form a hairpin structure characteristic of procaryotic transcription terminators (Platt. 1986).

The DNA encoding *exoX* was located 600 bp to the left of *exoY* (Fig. 4.8). One very likely ORF of 96 amino acids (Fig. 4.10), was found in this region. The *exoX* gene is transcribed divergently from *exoY* and hence the sense strand for *exoX* is the bottom strand depicted in figure 4.10. The coding region begins with an ATG at nucleotide position 348 and ends at position 57 (Fig. 4.10). The Fickett's TESTCODE program indicates a high level of non-randomness associated with the DNA sequence encoding *exoX* (Fig. 4.11E) and the proposed reading frame was the same as that declared most likely by Staden's "Positional Base Preference" program (represented as the top reading frame of Fig. 4.11D).

4.2.9 Secondary Structures of *exoX*, *exoY* and ORF1 and a Comparison with *R. l.* bv. *phaseoli exo* Genes

The only other *Rhizobium exo* genes that have been sequenced are two regions from *R. l.* bv. *phaseoli* and the sequences for these were published during the course of this research project. These two genes are *psi* (Borthakur and Johnston. 1987) and *pss* (Borthakur *et al.*, 1988). The predicted *exoX* and *psi* gene products are similar and the predicted product from *exoY* is similar to that of the second ORF in the *pss* operon (designated from here on as *pss2*).

ExoY is a polypeptide of 226 amino acids in length with an estimated molecular weight of 25,000 MW. This predicted polypeptide has a run of 24 entirely hydrophobic amino acids starting at residue 34, followed immediately by a lengthy 65 residue hydrophilic domain. The hydrophobic stretch is long enough to form a trans-membrane region or to associate by hydrophobic interactions with other protein domains (Oliver. 1987). A comparison of *exoY* with the *R. l.* bv. *phaseoli* gene, *pss2* (Borthakur *et al.*, 1988),

which encodes a 200 amino acid peptide (22,300 MW), showed that a high degree of similarity exists at the peptide level. The two putative proteins are 52% similar with 32% of the homology due to exact matches (Fig. 4.12A). Their similarity at the secondary structural level is also high, as demonstrated by the near superimposable hydrophobicity plots for ExoY and Pss2 (Fig. 4.12B). The probable regions of α helix, β sheet and turn tetrapeptides were predicted using Weinman's (1986) secondary structure program. The result for the *exoY* polypeptide is shown in figure 4.13A. The same program was used to analyze probable secondary structures for the *pss2* polypeptide and the five most probable tetrapeptides involved in turns, corresponded, in location, to five out of the seven most probable turn tetrapeptides for the *exoY* polypeptide (Fig. 4.12A). These five tetrapeptide sequences also shared some homology between the two proposed proteins at the amino acid level, where as the two extra tetrapeptide sequences for ExoY did not share any homology with Pss2 at the corresponding locations (Fig. 4.12A). Despite the similarities of these two deduced polypeptides, the surrounding DNA and ORFs do not share any homology. The phenotypes associated with *exoY* and *pss2* are also similar, suggesting that the relatedness of the two genes is due to their common function.

ExoX is a polypeptide of 96 amino acids in length. The first 55 residues are all hydrophobic except for one, and the remaining carboxy terminal region is hydrophilic in nature. The hydrophobicity plot for the *exoX* polypeptide (Fig. 4.14A) is shown alongside a hydrophobicity plot for the *psi* protein (Borthakur and Johnston. 1987) (Fig. 4.14B). The similarity between these two plots is striking. Both proteins are very hydrophobic for the amino half and then rapidly make the transition to a hydrophilic nature for the remaining half. In addition, both proteins are similar in size (96 amino acids for ExoX and 86 amino acids for Psi) and predicted molecular weights (approximately 10,500 MW and 9,500 MW respectively). The similarity between these two proteins is less apparent at the primary amino acid level (Fig. 4.14C). There is

Fig. 4.12 Comparisons between deduced polypeptide sequences of *exoY* and *pss2*. (A) Best alignment between the deduced protein sequences of *exoY* and *pss2*. Exact amino acid matches are indicated by an unbroken line, and functionally similar amino acids are indicated by a broken line. Amino acids have been grouped into the following families: acidic and amidic (D, E, N, Q), basic (H, K, R), polar (A, G, P, S, T), nonpolar (I, L, M, V), aromatic (F, W, Y), cysteine (C). Those tetrapeptide sequences that have a high likelihood [$P_t \geq 2.1 \times 10^{-4}$, (Chou and Fasman, 1978b)] of forming a turn in the peptide are enclosed within boxes.

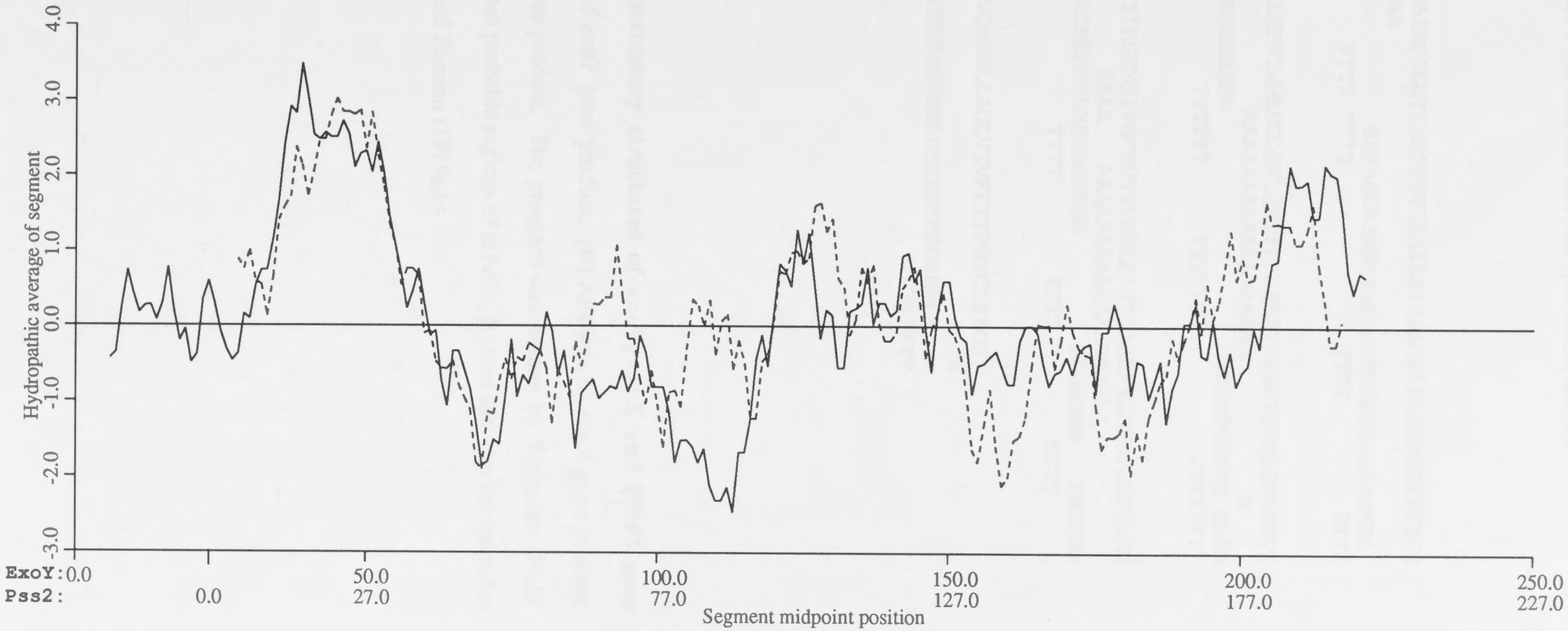
(B) The hydrophobicity plots of ExoY (unbroken line) and Pss2 (dotted line) are shown. Both plots were generated using a hydropathy program with the values of Kyte and Doolittle (1982) and written by Weinman (Ph.D. Thesis, 1986). The values on the horizontal axis represent the amino acid residue position and the values on the vertical axis represent the hydropathic average of a 11 residue segment of the polypeptide.

Fig. 4.12

A

```
ExoY: MKSATRSATTAFFIPQETGAIRPIGGISKRSFDVLIAILALIALSPLFLLVMGLVKFSDGGSIFYGHRRIGHNGQTFKCLKFRTMMENGDRVLQEFFKSNPAAYEEWRTTRKLQDDPRV
Pss2:                          VDLVLKRAFDIFSSLSALLVLAPFLLFVALLIKLDSPGPVLFKQTRWGKNCKAIKVYKFRSMRTDLCDVSGVAQTVKN----------DPRI

ExoY: TVVGSVLRKLSLDELPQLLNIIRGEMSIVGPRPVVEDELELYDSAAEFYLRS------RPGLTGLWQISGRNDVSYAT-----RVAFDTHYVQNWSLLADLVIVFKTIPAVCLSRGSY
Pss2: TRIGAILRRTNVDELPQLLNVLLGHMSVVGPRCHAIGMRAGGMLYEELVPEYHQRHAMRPGMTGLAQMRGLRGPTDRPAKARARIASDLYYVGNFSIVMDMRIIFGTVVSELTRGKGF
```

B



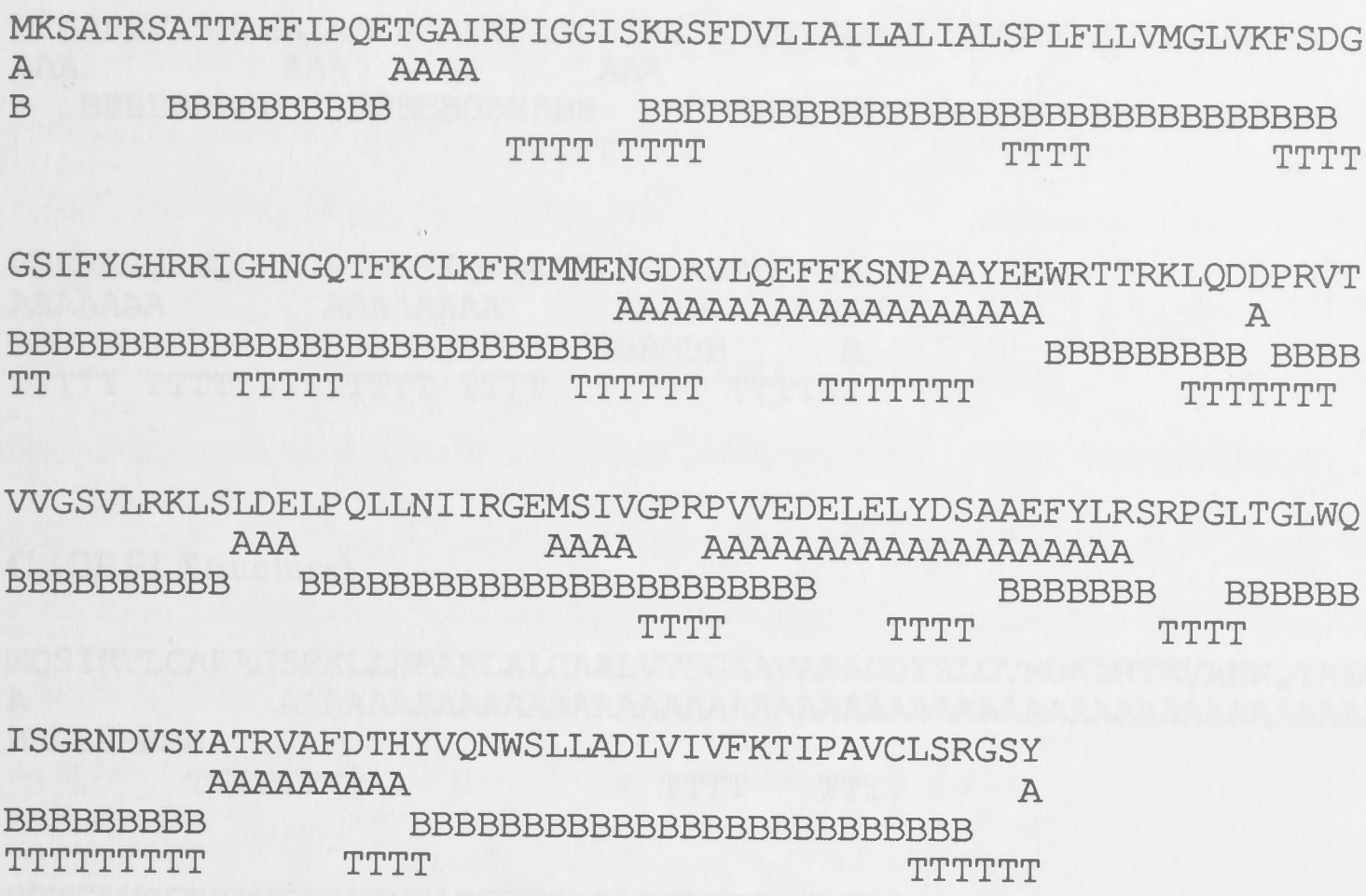


Fig. 4.13 Predicted secondary structures of *exoY*, *exoX* and ORF1 gene products. (A) Analysis of *exoY* gene product. (B) Analysis of *exoX* gene product. (C) Analysis of ORF1 gene product. The program was written by Weinman (Ph.D. Thesis, 1986) and determines probable regions of α helix, β sheet and turn tetrapeptides using algorithms of Chou and Fasman (1978a,b).

B (ExoX Structure)

```
MFAPRFVVSMLGALAAFAIATYFLTGSIASTAVQTLLCAVLIQVGYFLAVLFLVWKEA
AAA         AAA             AAA
   BBBBBBBBB   BBBBBBBBBBB   BBBBBBBBBBBBBBBBBBBBBBBBBBBBBB
                    TTTT

RDRRKLSPGQLPADPTNDEKQTGKLSLRRLNRPPHFNS
AAAAAAA       AAAAAAAA              A
                           BBBBBB     B
 TTTT TTTT TTTTTTTT TTTT         TTTTTT
```

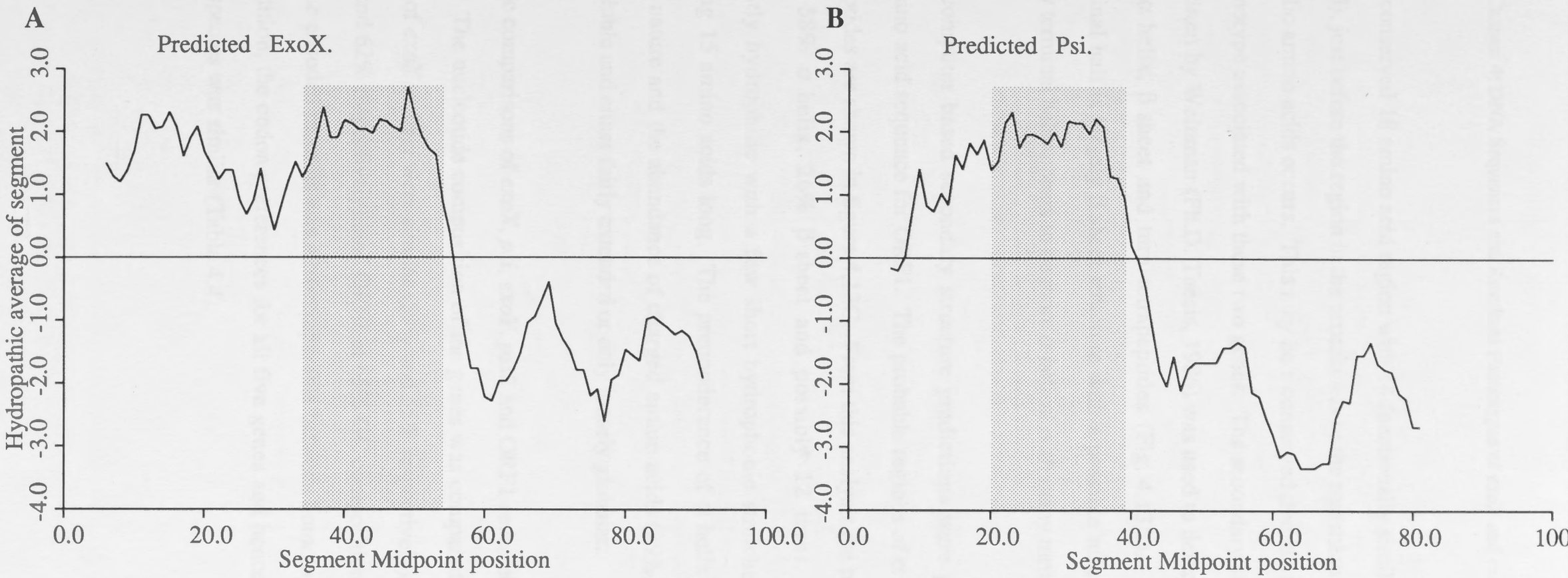
C (ORF1 Structure)

```
MQSIRVLGAPAGSRKLLHFARLALCAALVVSGAAVARADDYRLGVMDKLRVRVAEWQTAEGAV
A          AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
BBBBBBBBB
         TTTTTTT                 TTTT   TTTT

RDWSAVSGEYTVGASGSVSLPFVGDLPASGRTTTEVAEEIGIKMQKLFGLRDRPSASVEMAQY
AAAAAAA                              AAAAAAAAAAAAAAAAAAAAA AAAAAAAAAA
  BBBBBBBBBBBBB    BBBBBBB                                       BBBB
     TTTTT  TTTTT          TTTTTT                          TTTT


RPVYLYGEVETPGEYPYAPNLTVLKAVSLSGGLRRGPTGQRFARDYIAANGDSSVQVAERNRL
A                                                AAAAAAAAAAAAAA  AAAAA
BBBBBBB              BBBBBBBBBBB        BB                BBBBBB BBBB
           TTTTTTTTTTT         TTTTTTTTTTTT   TTTT TTTTTTTT   TTTT


LIRRARLQAEIAKHDKIELPEELKNAPGVDKLLESETALMVSRDKRQDRQLDALADLKSLLQS
        AAAAAAAAAAAAAAAAAA  AAAAAAAA         AAAAAAAAAAAAAAAAAAA
BBBBBBBB                                BBBBBBBB
                              TTTTTT           TTTTTTTT


EIESLAKKAETQQRQLELAMEDRDKVDSLAEKGLALSQRKLSLEQRVADVQSQLLDIDTN
AAAAAAAAAA        AAAAAAAAAAAAAAAAAAAAAAAAAAAAA
           BBBBBBB                                  BBBBBBBBBBBB
                         TTTT            TTTT
```

Fig. 4.13 Continued

Fig. 4.14 Comparisons between deduced polypeptide sequences of *exoX* and *psi*. The hydrophobicity plots of ExoX (**A**) and Psi (**B**) are shown. Both plots were generated using a hydropathy program with the values of Kyte and Doolittle (1982) and written by Weinman (Ph.D. Thesis, 1986). The values on the horizontal axis represent the amino acid residue position and the values on the vertical axis represent the hydropathic average of a 11 residue segment of the polypeptide. (**C**) Best alignment between the deduced protein sequences ExoX and Psi, without the introduction of any gaps. Exact amino acid matches are indicated by an unbroken line and functionally similar amino acids are indicated by a broken line. The region of highest homology is enclosed within a box corresponding to the shaded regions of A and B. Amino acids have been grouped into the following families: acidic and amidic (D, E, N, Q), basic (H, K, R), polar (A, G, P, S, T), nonpolar (I, L, M, V), aromatic (F, W, Y), cysteine (C).

Fig. 4.14



C

```
predicted ExoX
MFAPRFVVSMLGALAAFAIATYFLTGSIASTAVQ TLLCAVLIQVGYFLAVLF LVWKEARDRRKLSPGQLPADPTNDEKQTGKLSLRRLNRPPHFNS
                                    VHQRCFGLRASLSIFKAFAV TLAASVFLQVVYFLSLLF MSFRPTRESDRSIHSGTRQADQPQKRDRDKTEQSNVPKLDPRRKRRTP
predicted Psi
```

however, a conserved 18 amino acid region with 14 functionally similar amino acids (10 are identical), just before the region in the protein where the transition from hydrophobic to hydrophilic amino acids occurs. This may be a conserved domain responsible for the similar phenotype associated with these two genes. The secondary structure prediction program written by Weinman (Ph.D. Thesis, 1986) was used to determine the probable regions of α helix, β sheet and turn tetrapeptides (Fig. 4.13B). The hydrophobic amino terminal half is largely β sheet structure with a possible turn centrally located. The carboxy terminal half appears to be more α helical with many turns in the peptide.

The same computer based secondary structure predictions were performed on the deduced amino acid sequence for ORF1. The probable regions of α helix, β sheet and turn tetrapeptides are shown in figure 4.13C. From this analysis the protein is predicted to contain 58% α helix, 26% β sheet and possibly 12 turns. The peptide is predominantly hydrophilic with a few short hydrophobic stretches (Fig. 4.15), the longest being 15 amino acids long. The preponderance of α helix domains, largely hydrophilic nature and the abundance of charged amino acids (29%), suggest that the protein is soluble and either fairly extended or only loosely globular.

Further basic comparisons of *exoX, psi, exoY, pss2* and ORF1 revealed some interesting similarities. The nucleotide composition of the genes was compared (Table 4.2). The GC content of *exoX* and *psi* were similar (59% and 55% respectively), and for *exoY* and *pss2* (60% and 62% respectively), and ORF1 at 65% GC composition. The amino acid content of the encoded proteins was also comparable between homologous genes (Table 4.3). In addition, the codon preferences for all five genes and hence between the two *Rhizobium* species was similar (Table 4.4).

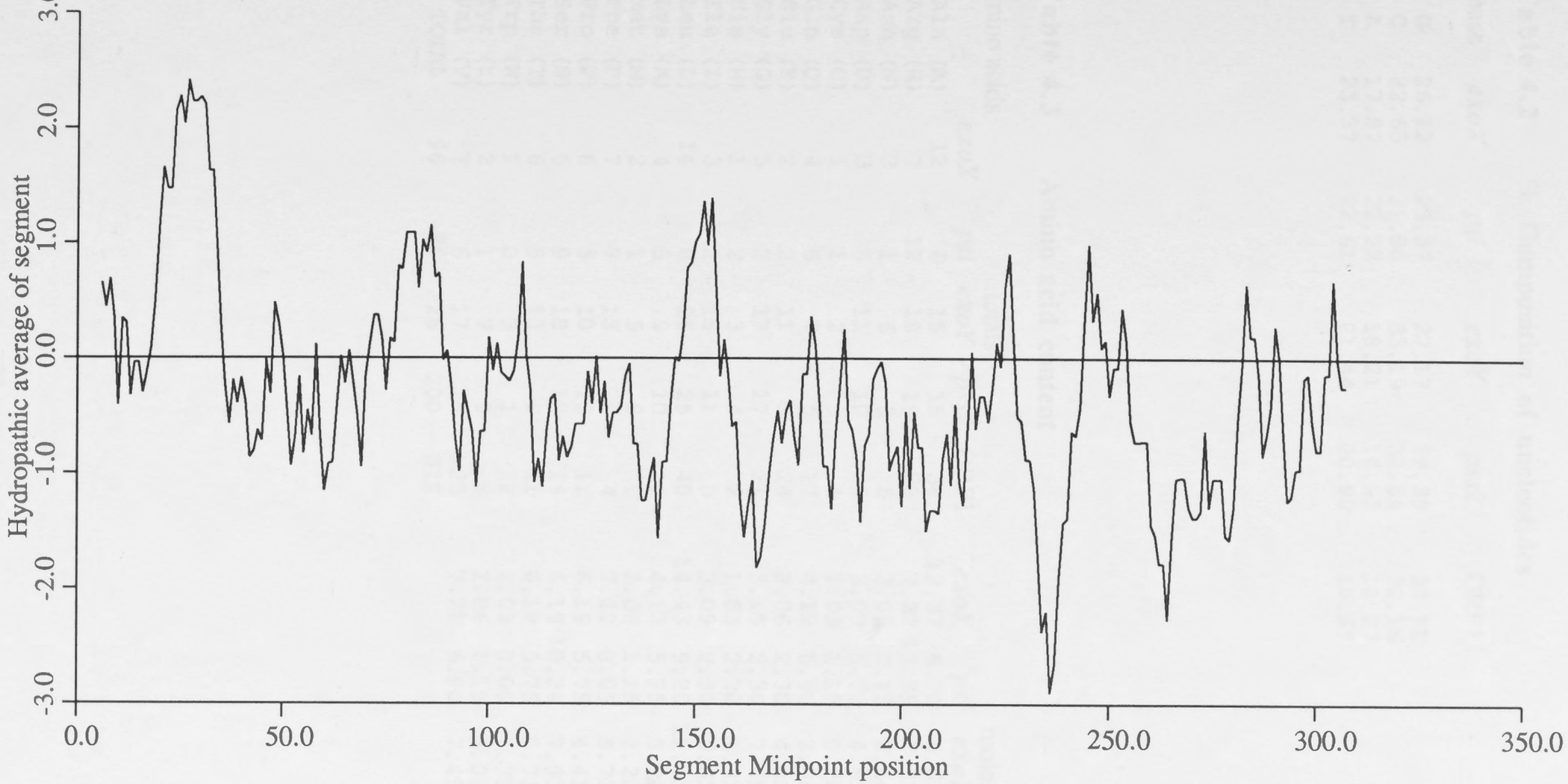


Fig. 4.15 The hydrophobicity plot of ORF1 predicted polypeptide.

The plot was generated by using a hydropathy program with the values of Kyte and Doolittle (1982) and written by Weinmann (1986). The values on the horizontal axis represent the amino acid residue positions, and the values on the vertical axis represent the hydropathic averages of an 11 residue segment of the polypeptide.

Table 4.2 % Composition of nucleotides

base	*exoX*	*psi*	*exoY*	*pss2*	ORF1
G	26.12	23.37	27.17	29.35	32.91
C	32.65	31.80	33.19	32.84	32.16
A	17.87	22.22	18.21	16.92	18.27
T	23.37	22.61	21.44	20.90	16.67

Table 4.3 Amino acid content

amino acids	totals					moles %				
	exoX	*psi*	*exoY*	*pss2*	ORF1	*exoX*	*psi*	*exoY*	*pss2*	ORF1
Ala (A)	12	6	15	15	38	12.37	6.90	6.61	7.46	12.18
Arg (R)	7	12	18	19	28	7.22	13.79	7.93	9.45	8.97
Asn (N)	3	1	6	5	5	3.09	1.15	2.64	2.49	1.60
Asp (D)	3	5	11	10	20	3.09	5.75	4.85	4.98	6.41
Cys (C)	1	1	2	3	1	1.03	1.15	0.88	1.49	0.32
Gln (Q)	4	6	7	5	17	4.12	6.90	3.08	2.49	5.45
Glu (E)	2	2	11	5	24	2.06	2.30	4.85	2.49	7.69
Gly (G)	5	2	17	17	21	5.15	2.30	7.49	8.46	6.73
His (H)	1	2	3	4	2	1.03	2.30	1.32	1.99	0.64
Ile (I)	3	2	15	11	9	3.09	2.30	6.61	5.47	2.88
Leu (L)	14	8	26	25	40	14.43	9.20	11.45	12.44	12.82
Lys (K)	4	5	9	10	16	4.12	5.75	3.96	4.98	5.13
Met (M)	2	1	5	9	6	2.06	1.15	2.20	4.48	1.92
Phe (F)	7	7	13	9	4	7.22	8.05	5.73	4.48	1.28
Pro (P)	6	5	10	10	11	6.19	5.75	4.41	4.98	3.53
Ser (S)	6	9	18	10	24	6.19	10.34	7.93	4.98	7.69
Thr (T)	6	5	13	9	11	6.19	5.75	5.73	4.48	3.53
Trp (W)	1	0	3	1	2	1.03	0.00	1.32	0.50	0.64
Tyr (Y)	2	1	7	5	8	2.06	1.15	3.08	2.49	2.56
Val (V)	7	6	17	18	25	7.22	6.90	7.49	8.96	8.01
TOTAL	96	86	226	200	312					

Table 4.4 Codon usage

codon	*exoX*	*psi*	*exoY*	*pss2*	ORF1	codon	*exoX*	*psi*	*exoY*	*pss2*	ORF1
GGG (G)	0	0	1	1	2	GCG (A)	4	2	2	3	10
GGA (G)	1	1	3	3	1	GCA (A)	2	1	2	1	5
GGC (G)	4	1	12	11	15	GCC (A)	5	2	10	11	22
GGT (G)	0	0	1	2	3	GCT (A)	1	1	1	0	1
AGG (R)	0	1	0	0	0	ACG (T)	3	1	5	3	8
AGA (R)	0	1	0	0	1	ACA (T)	1	1	0	0	1
AGC (S)	1	2	7	3	8	ACC (T)	2	2	6	5	2
AGT (S)	0	0	1	0	0	ACT (T)	0	1	2	1	0
CGG (R)	1	3	3	1	12	CCG (P)	2	0	6	9	3
CGA (R)	1	0	2	0	0	CCA (P)	1	2	0	0	0
CGC (R)	5	5	10	15	12	CCC (P)	2	2	3	1	5
CGT (R)	0	2	3	3	3	CCT (P)	1	1	1	0	3
TGG (W)	1	0	3	1	2	TCG (S)	1	3	6	5	11
TGA (*)	1	0	1	0	0	TCA (S)	1	1	0	0	0
TGC (C)	0	0	2	3	1	TCC (S)	3	3	4	2	4
TGT (C)	1	1	0	0	0	TCT (S)	0	0	0	0	1
GAG (E)	1	2	5	3	16	GTG (V)	3	1	4	6	3
GAA (E)	1	0	6	2	8	GTA (V)	0	1	0	1	1
GAC (D)	2	3	4	5	15	GTC (V)	3	2	11	10	13
GAT (D)	1	2	7	5	5	GTT (V)	1	2	2	1	8
AAG (K)	3	1	9	9	15	ATG (M)	2	1	5	9	6
AAA (K)	1	4	0	1	1	ATA (I)	1	1	0	1	2
AAC (N)	1	1	5	3	2	ATC (I)	2	1	11	8	5
AAT (N)	2	0	1	2	3	ATT (I)	0	0	4	2	2
CAG (Q)	3	5	7	5	15	CTG (L)	5	1	9	10	15
CAA (Q)	1	1	0	0	2	CTA (L)	1	2	0	0	1
CAC (H)	1	1	1	1	0	CTC (L)	2	0	13	9	18
CAT (H)	0	1	2	3	2	CTT (L)	3	3	4	2	4
TAG (*)	0	1	0	0	0	TTG (L)	2	1	0	4	2
TAA (*)	0	0	0	1	0	TTA (L)	1	1	0	0	0
TAC (Y)	0	0	4	4	4	TTC (F)	4	6	11	7	4
TAT (Y)	2	1	3	1	4	TTT (F)	3	1	2	2	0

4.2.10 The *exoY*::Tn*5* Mutation is Polar to ORF1

The deleterious nature associated with pJG22, when transferred into strain ANU2811, may have been due to the concurrent loss of other down stream genes (such as ORF1) by the same Tn*5* insertion. This was investigated by isolating pJG51::Tn*5* plasmids by a procedure identical to that used to isolate pJG22::Tn*5* plasmids (see 4.2.3). Plasmid pJG51 (Fig. 4.8) encodes the entire *exoY* gene and part of ORF1. The 3' end of the fragment, which maps within the ORF1 coding region, is transcriptionally fused to the *E. coli lacZ* gene. Plasmid pJG51 has a similar reduced transfer efficiency as pJG22 and also resulted in Exo$^+$ and Exo$^-$ colony morphologies of ANU2811(pJG51) transconjugants. Exo$^-$ ANU2811(pJG51) transconjugants were the result of a homologous recombination event generating the plasmid pJG51::Tn*5* (Fig. 4.4), while the plasmids carried by Exo$^+$ ANU2811(pJG51) transconjugants appeared unalterred. Transcriptional activities of these ORF1'-*lacZ*$^+$ fusions were compared. β-galactosidase activity from the fusion on pJG51 was fairly high while the fusion on pJG51::Tn*5* had no activity (Table 4.5). It was concluded that the 2811::Tn*5* insertion was polar to all down-stream genes in the operon. Furthermore, this observation supported data that suggested that ORF1 is part of the same transcriptional unit as *exoY*.

4.2.11 Promoter Mapping by S1 Nuclease and *lacZ* Fusions

After analysis of the nucleotide sequence, no satisfactory ORF could be assigned to the DNA sequence occurring between *exoX* and *exoY*. It was not clear whether this intervening DNA sequence was an extensive 5' untranslated region of either the putative *exoY*-ORF1 operon or the *exoX* operon or a separate transcriptional unit. Potential transcriptional units in this region were mapped with S1 nuclease and by fusions to the *E. coli lacZ* gene. Two single stranded DNA probes were able to be protected by mRNA from S1 nuclease. The results are shown in figure 4.16 and diagrammatically represented in figures 4.8 and 4.10. The cluster of bands in lane 3 of figure 4.16 suggests that this intervening sequence is transcribed divergently from *exoX*. The length

Table 4.5 β-Galactosidase activity of *lacZ* fusions in the wild-type background.

Plasmid	Construction	Activity[a]	S.D.
pMP220	Vector	139	26
pJG54	*exoY'-lacZ*$^+$	1881	302
pJG60	*exoX'-lacZ*$^+$	1575	84
pJG70	990bp *Nru*I-*lacZ*$^+$	28	7
pJG51	ORF1'-*lacZ*$^+$	936	22
pJG51::Tn*5*	ORF1'-*lacZ*$^+$	3	0.8

[a] Activity units are as defined by Miller (1972).

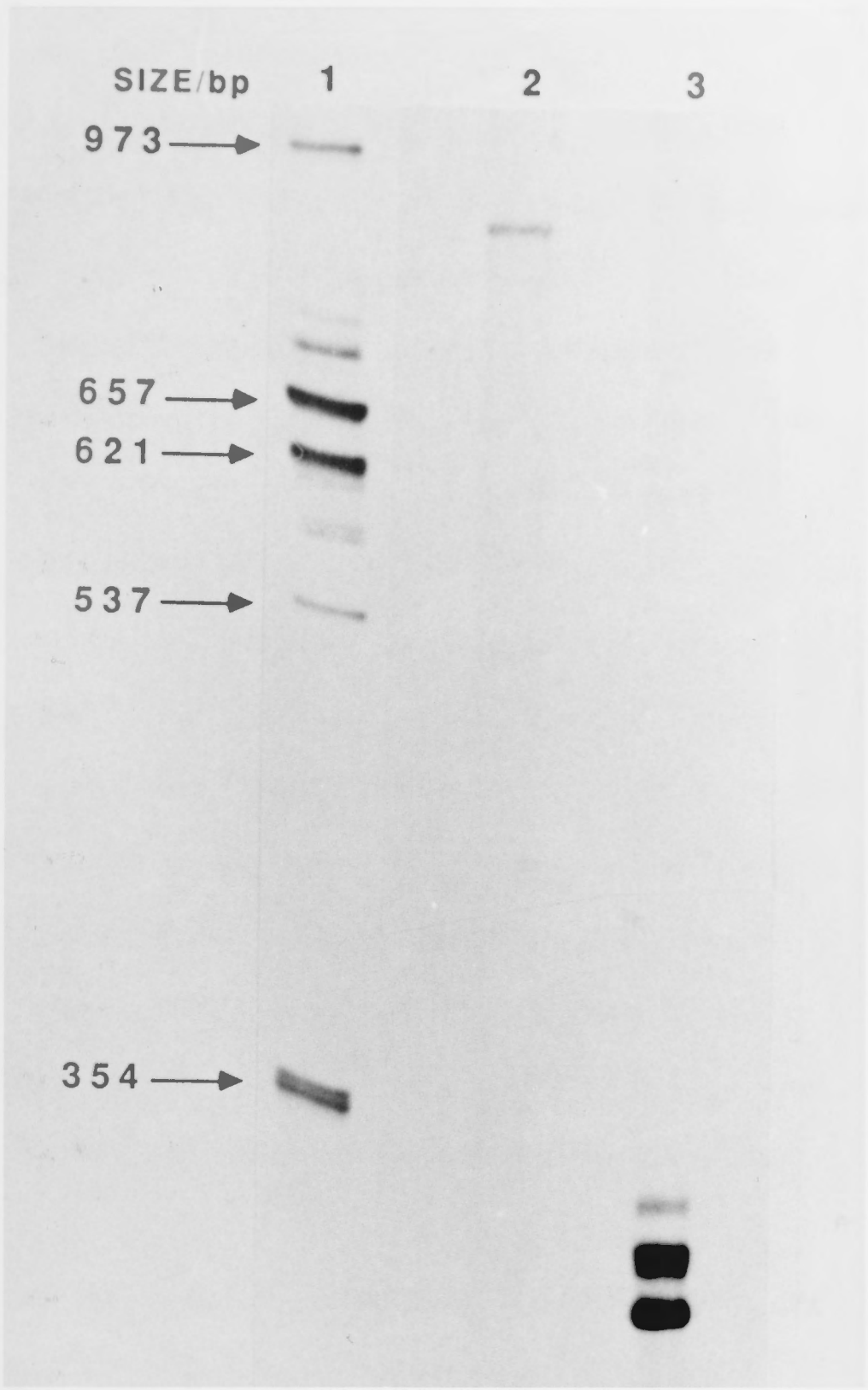


Fig. 4.16 Autoradiograph showing the protected probe DNA after S1 digestion. Lane 1 is *Cla*I digested λ DNA with the sizes of the authentic bands indicated by arrows. Lane 2 shows the length of single stranded probe DNA from the 5' ^{32}P-labelled *Sma*I site (at nucleotide position 1700, within the *exoY* coding region), which was protected by mRNA. Lane 3 shows the lengths of probe DNA from the 5' ^{32}P-labelled *Eco*RI site (at nucleotide position 789, within the untranslated, transcribed region), which was protected by mRNA. The two probes used in lanes 2 and 3 had a common 3' unlabeled *Cla*I site 2.2kb from the *Sma*I site (lane 2) and 1.3kb from the *Eco*RI site (lane 3).

of single stranded probe DNA from the 5' ^{32}P-labelled *Eco*RI site (at nucleotide position 789, within the untranslated, transcribed region), that was protected by mRNA was approximately 320 bp. This places the transcription start position around nucleotide position 470. A transcriptional fusion to *lacZ* (pJG70) using the promoterless vector pMP220 showed no activity (Table 4.5), and this demonstrated that transcription of this region terminates upstream of the *Nru*I site at nucleotide position 989 (Fig. 4.10).

The second single stranded DNA probe was 5' ^{32}P-labelled at the *Sma*I site at position 1700 (within the *exoY* coding region) and gave a clear band from a protected fragment approximately 640 bp in length (Fig. 4.16, lane 2). This demonstrated that the transcription initiation site for *exoY* was around nucleotide position 1060, which is downstream of the *Nru*I site at position 989 . An *exoY'-lacZ*$^{+}$ fusion (pJG54) at the *Pst*I site at nucleotide position 1408 (Fig. 4.10), shows significant *lacZ* activity (Table 4.5) and confirmed that the direction of transcription is from left to right (Fig. 4.8). Strong activity of an *exoX'-lacZ*$^{+}$ fusion (pJG60), (Table 4.5) at the *Nru*I site at nucleotide position 290 (Fig. 4.10), indicated that *exoX* was transcribed divergently from *exoY*.

4.2.12 Identification of Possible Transcription and Translation Initiation Signals.

The putative ribosome binding site (RBS) for *exoY* is 5'-TGGAGT-3' and this is identical to the presumptive RBS for *R. meliloti nodA* (Török *et al.*, 1984) and is similar (four out of six nucleotides match) to those of *nodD* and *nodH* (Egelhoff *et al.*, 1985; Fisher *et al.*, 1987). S1 promoter mapping experiments indicated that the start of transcription of *exoY* was approximately 70bp upstream of the putative start codon. Upstream of this position, corresponding with the $^{-}$35 position, was the sequence 5'-CTGCCA-3'; this had four out of six matches with the possible *R. meliloti nodA, nodF* and *nodH* $^{-}$35 sequences (Fisher *et al.*, 1987 and 1988). An optimal 17bp spacing existed between this $^{-}$35 sequence and a downstream potential $^{-}$10 sequence, with four

out of six matches to the procaryote consensus sequence (McClure, 1985) or five out of six matches to a presumptive *nodD* $^{-}$10 sequence (Fisher *et al.* 1988). The putative *exoX* RBS, 5'-AGGCGG-3' had a five out of six matches with the procaryote consensus sequence (Gold *et al.*, 1981). Virtually consensus-like (McClure, 1985) promoter sequences [5'-TTGAag-(17bp)-TATAgT-3'] exist 50bp upstream of the proposed *exoX* start codon. The nucleotide sequence was also scanned for promoter-like sequences resembling those for Ntr regulated *nif* gene promoters (Beynon *et al.*, 1983); no sequences were identified that were similar to the proposed consensus.

4.3 DISCUSSION

In this chapter, the nucleotide sequence for 2,800 bp of DNA involved in the synthesis of EPS for *Rhizobium* sp. strain NGR234 was presented. To assist in assigning transcriptional units and genes to the ORFs, a combination of S1 promoter mapping, *lacZ* transcriptional fusion experiments, and analysis of the phenotypes associated with subcloned regions was employed to complement the DNA sequencing and its computer analysis.

The Exo^{-} mutants used in this study resulted from single Tn*5* insertions into the wild type-genome of strain ANU280 and the locations of the mutations were mapped to specific *Eco*RI fragments (Chapter 3). R-prime plasmids carrying mutations in *exoY* were used to define two types of strain ANU280 transconjugants with repressed EPS synthesis. The introduction to strain ANU280 of R-prime plasmids carrying Tn*5* insertions corresponding to the *exoY11*::Tn*5* or *exoY90*::Tn*5* alleles resulted in Exo^{-} colonies (persistent dominant phenotype) and R-prime plasmids carrying Tn*5* insertions corresponding to the *exoY08*::Tn*5* or *exoY40*::Tn*5* alleles conferred an Exo^{-} phenotype on strain ANU280, which upon prolonged incubation resulted in Exo^{+} colonies (leaky dominant phenotype) (Chen, 1987, Ph.D. Thesis). All four Tn*5* insertion sites were located within *exoY*. The two insertion sites for the "persistent-dominant" mutant alleles

were at amino acid residue positions 20 (2811::Tn*5*) and 124 (2890::Tn*5*), and the two insertion sites for the "leaky-dominant" alleles were at positions 161 (2808::Tn*5*) and 202 (2840::Tn*5*). Whatever the function of ExoY may be, it is evident that it has some wild-type activity when at least 161 of the total 226 amino acids have been translated.

The production of EPS by NGR234 appears to be strictly controlled by the products of *exoY* and *exoX*. The evidence suggests that the gene product of *exoX* is a repressor of EPS synthesis, because an elevated copy number of this gene results in the inhibition of EPS synthesis by *Rhizobium* sp. When *exoX* is carried on an IncP1 plasmid in the absence of *exoY*, the phenotype of the strain ANU280 merodiploid transconjugants is Exo$^-$. Normal EPS production occurs when the copy number of *exoY* is increased to a level equal to that of *exoX*; cloned fragments carrying both of these genes do not confer the Exo$^-$ phenotype on strain ANU280 transconjugants. Since normal EPS synthesis is sensitive to slight elevations of the copy number of *exoX* relative to *exoY*, this suggests the possibility of an interaction either between the products of the two genes or the product of *exoY* and the promoter of *exoX*. Conditions favouring repressed expression of *exoY* or enhanced expression of *exoX* in the wild-type strain might lead to repression of EPS production.

Transposon Tn*5* insertions into the genomic copy of *exoY* abolish the ability of strain ANU280 to synthesize acidic EPS (Chen *et al.*, 1985). This Exo$^-$ phenotype was probably due to the presence of *exoX* unchecked by *exoY* rather than the mutation of *exoY* as a structural gene. Normal EPS production was restored by the introduction of fragments carrying the wild-type allele for *exoY*. Recombinant R-prime plasmids carrying wild-type *exoY* (with very low copy numbers of 3 per cell), will correct the Exo phenotype of *exoY*::Tn*5* mutants in 100% of the cases (Chen *et al.*, 1988). When the copy number of this wild-type *exoY* allele is increased on a recombinant IncP1 plasmid (copy number of 10 per cell) and transferred into *exoY*::Tn*5* mutants, the frequency of

correction to Exo$^+$ in the transconjugants was no longer 100%, but was 52%. The other 48% of the transconjugants remained Exo$^-$. In the Exo$^-$ transconjugants, a normally rare double reciprocal recombination event had occurred between the DNA flanking the genomic Tn*5* insertion and homologous *Rhizobium* DNA cloned on the plasmid. Therefore, an Exo$^-$ transconjugant has several copies of the nearby *exoX* on the plasmid and only a single copy of the wild-type *exoY* allele in the genome. This imbalance in favor of *exoX* results in the inhibition of EPS biosynthesis by these *Rhizobium* cells. The Exo$^+$ transconjugants of strain ANU2811 carrying pJG22, on the other hand, appear to have been complemented by the introduced fragment, because the plasmid has not been altered in any way. However, since these transconjugants were slow to appear, another explanation is that these cells have undergone a suppressor mutation elsewhere in the genome to compensate for the presence of extra copies of *exoY*. In conclusion, it appears that the presence of *exoY* at approximately 10 copies per cell is deleterious to the cell growth of *exoY*::Tn*5* mutants. This is supported by the strong selection for wild-type *Rhizobium* cells when merodiploid strains were passaged through *Leucaena* nodules. The reason why strain ANU280 is not affected in the same way by 10 copies of *exoY*, is not yet understood. One explanation is that the Tn*5* insertion into *exoY* is polar to a down stream gene that is not present on the cloned *Rhizobium* DNA of pJG22. Possibly, the absence of this putative gene creates an intolerance to high levels of *exoY*. The much larger cloned inserts of the R-prime plasmids would contain the whole *exoY*-ORF1 operon. However, elements on the 10 kb *Bam*HI fragment are definitely lethal in strain ANU280, as well as other mutants, when cloned on IncQ vectors, presumably due to the very high copy number afforded by this replicon.

Interestingly, wild-type *exoY* carried by pJG22 affected the viability of transconjugants when transferred into some mutants, but not others (Table 4.1). Those mutants affected by pJG22 included *exoY*::Tn*5* mutants (also lacking ORF1) and Tn*5* mutants from two other Exo$^-$ genetic complementation groups (B and G) and also a Tn*5* mutant that over

produces exopolysaccharide (ANU2895). In strain ANU2895 a Tn*5* is believed to have inserted within a gene that is a negative regulator of EPS synthesis, thus resulting in the over production of EPS (Chen, 1987, Ph.D. Thesis). This common intolerance to elevated levels of *exoY* possibly indicates a cooperative involvement of these genes in the synthesis of EPS. Perhaps, *exoY*, loci A and G, and the allele mutated in ANU2895, may all encode components or regulatory molecules that participate in a single EPS processing complex or biosynthetic pathway for an EPS precursor molecule; such that when anyone of these genes are absent and *exoY* levels are increased, the results of the altered complex or pathway are deleterious to the cell's viability. Those *Rhizobium* cells that are viable may carry a secondary suppressor mutation that has allowed them to adapt. Attempts were made to cure ANU2811(pJG22) transconjugants of their plasmids in order to study these putative suppressor mutations further. Strains that were successfully cured of their introduced IncP1 plasmids had suffered either extensive deletions in the *exo* region (in the case of Exo$^-$ transconjugants) or recombination events resulting in the loss of the Tn*5* insertion (in the case of Exo$^+$ transconjugants). In order to better understand these observations, more information about the other genes involved is required. However, it is clear that *Rhizobium* cells can be sensitive to high levels of *exoY* when they are lacking certain other *exo* genes.

The phenotype associated with multiple copies of *exoX* is the same as that already reported for the *R. l.* bv. *phaseoli* gene *psi* (Borthakur *et al.*, 1985). The proposed Psi polypeptide is 86 amino acids (Borthakur and Johnston, 1987) and this is similar to the proposed 96 amino acid ExoX polypeptide. In addition, the hydrophobicity plots for these proteins are strikingly similar. At the primary sequence level however, there is less similarity between the proteins encoded by *exoX* and *psi*, except for an 18 amino acid domain, where 14 of the residues are functionally similar (10 are identical). It is possible that *exoX* and *psi* are related genes, where the only evolutionary constraints have been within the 18 amino acid domain and the overall tertiary structure of the protein (*ie.*

maintaining a hydrophobic amino-terminal half and a hydrophilic carboxy-terminal half). It is possible that the hydrophobic amino-terminal region is inserted into the membrane, as already suggested for Psi (Borthakur and Johnston, 1987). Alternatively, this hydrophobic region may associate, by hydrophobic interactions, with other protein subunits to form a multimeric complex. The length of the hydrophobic region (55 amino acids for ExoX) suggests that both possibilities are plausible. The homologous domain between proteins encoded by *exoX* and *psi* occurs in the hydrophobic region just before the polypeptide makes a rapid hydrophilic transition. Therefore, the most amino-terminal 20 or so amino acids could form a trans-membrane signal peptide, which would still leave the conserved hydrophobic region available for association with hydrophobic domains of other proteins. One protein, which may be a candidate for this type of multimeric association, is that encoded by *exoY*, which has an internal hydrophobic region spanning 24 amino acids.

In the same way that *exoY* can counter the inhibitory effects of *exoX*, *pss* could counter the inhibitory effects caused by *psi* in *R. l.* bv. *phaseoli* (Borthakur *et al.*, 1988). Borthakur *et al.* (1988) reported the presence of two ORFs after sequencing the *pss* region. The second ORF, termed *pss2*, encoded a presumptive protein that demonstrated considerable homology to that of *exoY*. Both proteins are of similar size and molecular weight, 32% identical in their primary amino acid sequence, a further 20% of the amino acids are functionally conserved, their predicted secondary structures are very similar and their phenotypes are the same; the two genes are undoubtedly evolutionarily related. Despite their obvious relatedness, the polypeptide sequences have diverged quite significantly for proteins that perform the same function. Given that the structures of the EPS repeat units for *R. l.* bv. *phaseoli* (Philip-Hollingsworth *et al.*, 1989) and *Rhizobium* strain NGR234 (Djordjevic *et al.*, 1986) are different, then the divergence observed between *exoY* and *pss2* may reflect their different specificities for different oligosaccharide structures. In *R.* sp. NGR234, the coding regions for *exoX* and *exoY*

are separated by only 800 bp and the genes are not located on the symbiotic megaplasmid. In contrast, *psi* maps on the *R. l.* bv. *phaseoli* symbiotic megaplasmid (Borthakur *et al.*, 1985) and is totally unlinked to *pss*, which does not map on the symbiotic megaplasmid (Borthakur *et al.*, 1986). Up-stream (5') of the *pss2* gene is 1 kb of largely untranslated DNA; Borthakur *et al.* (1988) suggest that an ORF (*pss1*) of 58 amino acids may be translated. Similar to *pss2*, there is 800 bp of untranslated DNA 5' to the *exoY* coding region. However, S1 promoter mapping indicates that this up-stream DNA is a separate transcriptional unit to that of *exoY*; whereas *pss1* and *pss2* are in the the same complementation group, suggesting that they comprise a single operon (Borthakur *et al.*, 1988). Functions have not yet been elucidated for either of these 5' DNA regions.

Publication

The information presented in this chapter has resulted in the following refereed publication:-

Gray, J. X., M. A. Djordjevic, and B. G. Rolfe. 1990. Two genes that regulate exopolysaccharide production in *Rhizobium* sp. strain NGR234: DNA sequences and resultant phenotypes. *J. Bacteriol.* **172**:193-203.

CHAPTER FIVE

Regulation of *exoX* and *exoY*

5.1 INTRODUCTION

Overwhelming evidence indicates that genes responsible for EPS biosynthesis are essential for the successful symbiosis between rhizobia and their legume hosts in cases where the resulting nodule structure is of the indeterminate type (see section 1.6). However, results from several studies suggest that EPS may not be produced by *Rhizobium* cells in the symbiotic bacteroid state. An electron microscopical examination of the infection process in alfalfa (Jordan *et al.*, 1963) revealed that an electron dense material, believed to be EPS, was present in the infection thread matrix, but not around mature bacteroids. It was presumed that transformation into bacteroids was accompanied by the cessation of EPS production and that the conditions of the nodule may provide an environmental stimulus. It has been known for some time that the central tissue of a nodule is essentially anaerobic (Tjepkema and Yocum, 1974) and that a low O_2 tension is imperative for synthesis and activity of the nitrogenase enzyme (Bergersen *et al.*, 1976). More recently, the central regulatory component of *nif* gene expression, *nifA*, from *R. meliloti* was shown to be induced when the oxygen concentration is reduced to microaerobic levels (Ditta *et al.*, 1987).

Whether oxygen levels influence EPS production has been investigated by several authors. Electron microscopy was used to examine cells from core samples taken through colonies growing on soft agar medium (Pankurst and Craig, 1978). It was found that cells at the aerobic surface were surrounded by EPS, but the presence of EPS diminished as the cells analyzed came from deeper within the colony and at the anaerobic bottom EPS was absent. An investigation into EPS synthesis in *Bradyrhizobium japonicum* under anaerobic free-living conditions and during symbiosis was conducted by Tully and Terry (1985). Their wild-type strain, RT2, produced copious amounts of

EPS during aerobic growth in liquid culture, but their symbiotically effective Exo$^-$ mutant, RT176-1, produced about 12% of the amount of EPS produced by RT2. The amounts of EPS recovered from nodules induced by both of these strains on soybean were both approximately equal and both very low compared to the amount synthesized under free-living conditions. Furthermore, gas chromatographic analysis of the recovered EPS demonstrated that the sugar composition resembled that of EPS recovered from uninoculated root tissue and was very different from the chemical composition of EPS isolated from free-living liquid cultures. Their (Tully and Terry, 1985) conclusion was that the host plant tissue was the source of the EPS and that the *Rhizobium* cells did not produce EPS within the nodules. Measurements of EPS production by *B. japonicum* under aerobic and anaerobic liquid cultures demonstrated that although the doubling time of the anaerobic culture was increased by 50%, the amount of EPS synthesized was decreased by 92% (measurements were normalized with respect to cell density). Finally, Tully and Terry (1985) demonstrated that cells failing to synthesize adequate quantities of EPS were also inefficient at binding soybean lectin; suggesting that a requirement for EPS exists at the onset of nodulation. In the case of *B. japonicum*, reduced O_2 tension within the plant may result in the apparent repression of EPS production.

Apart from oxygen tension, a range of other physical conditions may act as stimuli for EPS regulation; these include: availability and source of nitrogen, pH, carbon sources, and other nutrients. These have all been shown to affect EPS production by bacteria grown in liquid culture, albeit differently from one bacterial species to another (reviewed by Sutherland, 1982 and references therein). The *Rhizobium* cells would be expected to experience variations in these conditions as they enter the plant tissue from the surrounding soil. The plant has a rich supply of carbon in the forms of sugars, organic acids, amino acids and fermentation products (Streeter, 1981; Stumpf and Burris, 1979; Tajima and La Rue, 1982). However, most of these various forms of carbon are not

available to the bacteroids. Bacteroid cells are kept isolated from the plant cytoplasm by a membrane of plant origin termed the peribacteroid membrane (PBM). There are usually several bacteroids enclosed within a single envelope of PBM and this is referred to as a peribacteroid unit (PBU). It is presumed that the PBM's function is to regulate the passage of metabolites from the plant cell cytoplasm to the bacteroids within. It appears that the PBM is a selective permeability barrier for various forms of carbon. Price *et al.* (1987) measured the stimulated uptake of O_2 by PBUs in response to the addition of various forms of carbon and interpreted this stimulated bacteroid respiration as an ability for the carbon source to pass through the PBM and be metabolized by the bacteroids. The dicarboxylic acids succinate and malate induced the greatest stimulation of O_2 uptake and arabinose, pyruvate and oxoglutarate stimulated respiration poorly if at all. A dicarboxylate anion transporter is believed to be operating on the PBM of soybean root nodules (Udvardi *et al.*, 1988a) . The PBM is impermeable to L-glutamate, despite it being a dicarboxylic acid. However free bacteroids have a high affinity transport system for glutamate uptake (Udvardi *et al.*, 1988b). Thus, *in vivo*, the PBM would exclude L-glutamate as a source of carbon for the bacteroids (Udvardi *et al.*, 1988b). Since glutamate is also a form of fixed nitrogen, the plant would have evolved a system for blocking its passage through the PBM. Mutants defective in dicarboxylic acid transport (*dct*) have been isolated in *R. leguminosarum* bv. *viciae* (Finan *et al.*, 1983), *R. l.* bv. *trifolii* (Ronson *et al.*, 1981), and *R. meliloti* (Bolton *et al.*, 1986). These mutants are still capable of utilizing other carbon sources when free-living, but form ineffective nodules on their respective host plants. The results suggest that the capacity to utilize dicarboxylic acids by bacteroids is essential during symbiosis and that only in the bacteroid state are *Rhizobium* cells limited to dicarboxylic acids as their sole source of carbon.

The concentration gradient of fixed nitrogen that would inevitably occur within nodule tissue may also act as environmental stimuli regulating polysaccharide synthesis. Most of

the atmospheric nitrogen fixed by the bacteroids is released as NH_3 (Bergersen and Turner, 1967). Ammonia is toxic to cells and is assimilated very quickly by the plant into amino acids and other compounds (Boland *et al.*, 1980). The active uptake of ammonium ions by an ammonium permease can be induced in free-living *R. l.* bv. *viciae* (O'Hara *et al.*, 1985) and *B. japonicum* (Howitt *et al.*, 1986) under conditions of low nitrogen availability and is repressed by high concentrations of ammonia. However, there was no evidence of the ammonium permease in bacteroids isolated from peas (O'Hara *et al.*, 1985) or soybeans (Howitt *et al.*, 1986) and both sets of authors speculated that the ammonium products of N_2 fixation repressed ammonium permease genes and that during symbiosis, neutral NH_3 molecules diffuse from the bacteroid to the plant cytosol. Other *Rhizobium* genes have been found to be under the control of ammonium regulation. The inducibility of the early nodulation genes *nodABC* from *R. meliloti* strain Rm41 was found to be inhibited when the ammonium ion level was increased (Dusha *et al.*, 1989). Also in *R. meliloti*, two genes involved in glutamine biosynthesis, *glnII* and *glnT*, were repressed by high concentrations of ammonium or glutamine (de Bruijn *et al.*, 1989). In both of the above cases, the respective authors demonstrated that the regulation was transcriptional and involved the nitrogen regulatory genes *ntrA* and *ntrC*. In *B. japonicum* the *glnB* gene is transcriptionally regulated by the *ntrC* gene product and *glnB* expression was enhanced under conditions of ammonia starvation (Martin *et al.*, 1989). Furthermore,the expression of *glnB* in bacteroids and the continued involvement of NtrC in its expression, suggests that the ammonia levels perceived by bacteroids may be low (Martin *et al.*, 1989). This conflicted with the general belief that a fairly rich fixed nitrogen environment exists within the nodule (discussed by Brown and Dilworth, 1975). However, Martin *et al.* (1989) was measuring *glnB* mRNA levels from bacteria within nodule tissue, where as Brown and Dilworth (1975) were measuring enzymic activity of glutamine synthase. Martin *et al.* (1989) discussed the possibility that the fixed nitrogen levels perceived by the bacteroids is not as high as has been thought and cites a report that at least 94% of ammonia is

exported from bacteroids (O'Gara and Shanmugam, 1976). There have been no definitive studies to measure the levels of fixed nitrogen within the PBUs.

In this chapter, transcriptional fusions of *exo* gene promoters to a promoterless *E. coli* *lacZ* gene were used to investigate any influences that growth conditions had on the expression of these *exo* genes. The *lacZ* gene encodes the enzyme β-galactosidase, which hydrolyzes β-D-galactosides. Measurement of β-galactosidase activity using chromogenic substrates can be used as a relative measure of transcriptional activity from the test promoter. Figure 5.1 is a summary of the fusion constructs involving varying amounts of DNA from the *exoX* and *exoY* region that were analyzed. Another putative *exo* gene promoter was analyzed in parallel; this was a *lacZ* transcriptional fusion of a promoter in the vicinity of the Tn*5* insertion site for mutant ANU2895 (Fig. 5.2) (Arioli, 1987). Although the promoter was never proven to be that of the *exo* gene mutated in ANU2895, it was useful as a control because the DNA was not from the *exoX-exoY* region. Any effects on transcription of these promoters due to the sources of carbon or nitrogen, availability of oxygen or nitrogen and variations in pH, were investigated. The relative amounts of EPS produced by *Rhizobium* sp. strain NGR234 at different stages of culture growth and when grown aerobically and anaerobically was also investigated. In addition, the activities of the promoters when expressed within different Tn*5* *exo* mutant backgrounds were analyzed as a possible means to identify *trans*-acting genes that regulate these *exo* promoters.

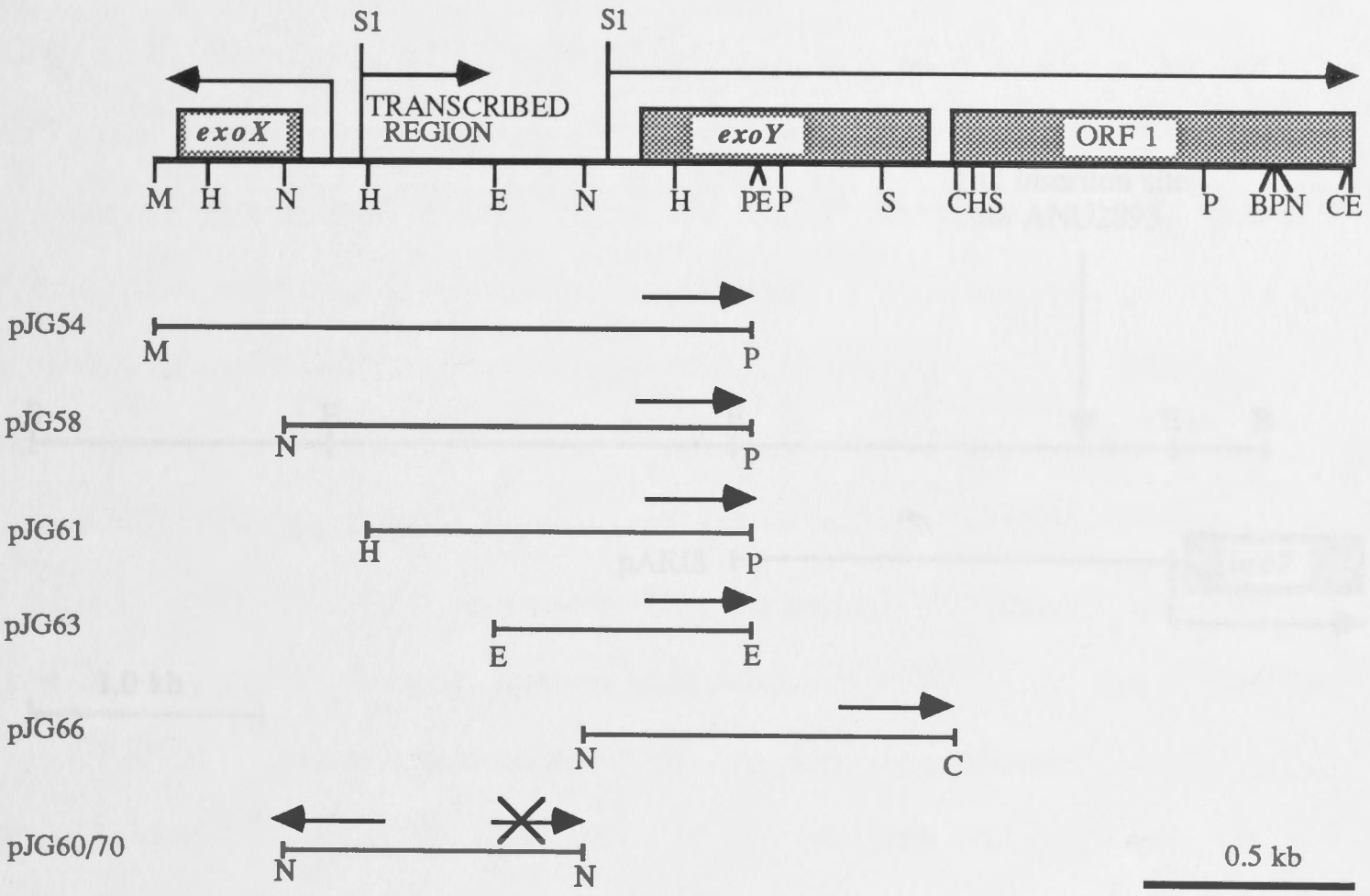


Fig. 5.1 Summary of the DNA fragments from the *exoX* and *exoY* region that have been cloned upstream of the promoterless *lacZ* gene of the vector pMP220. The names of recombinant plasmids, lengths of cloned DNA and insert orientations are all indicated. The fusion point is indicated by the arrow head and the direction of the arrow represents the direction of *lacZ* transcription. Symbols " ⟶ " and " ✕⟶ " indicate presence or absence (respectively) of β-galactosidase activity. Restriction sites are: C, *Cla*I; E, *Eco*RI; H, *Hind*III; M, *Mlu*I; N, *Nru*I; P, *Pst*I. A complete description of the genetic map is given in the legend of figure 4.8.

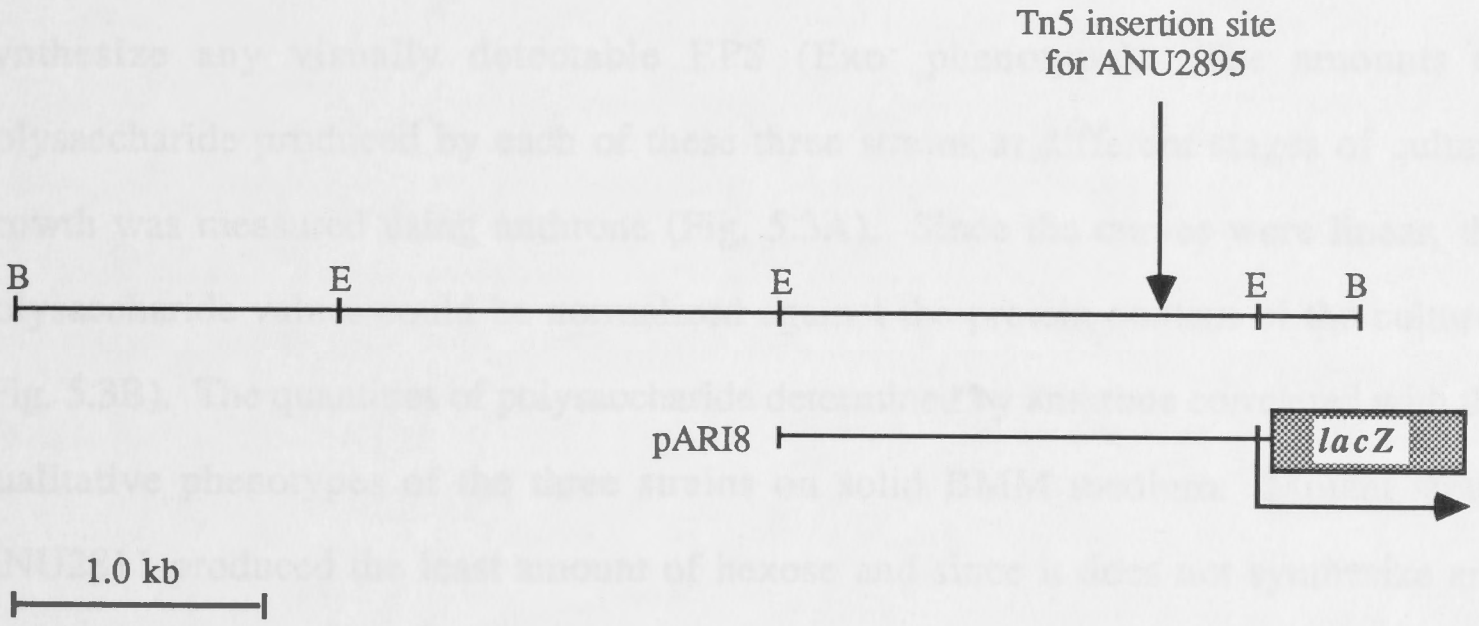


Fig. 5.2 Partial restriction map of the DNA region surrounding the Tn*5* insertion site of the Exo^{++} mutant ANU2895. The DNA from this region that has been cloned upstream of the promoterless *lacZ* gene of the vector pMP220 is illustrated (pARI7). The direction of the arrow represents the direction of *lacZ* transcription. Restriction sites are: B, *Bam*HI and E, *Eco*RI. The recombinant clone pARI8, and all of the information for this figure was provided by Tony Arioli (B.Sc. Honours Thesis, Australian National University, 1987).

5.2 RESULTS

5.2.1 Effectiveness of the Anthrone-H_2SO_4 Assay

The sensitivity of anthrone for the quantitative measurement of EPS in cultures where there will also be low concentrations of other polysaccharides such as LPS, cyclic β-1,2-glucan and other endogenous glycogen sources, was examined. The three *Rhizobium* strains used were: ANU280, which is the wild-type strain and produces a normal level of EPS (Exo^+ phenotype); ANU2895, which is a mutant of ANU280 that overproduces EPS (Exo^{++} phenotype); ANU2811, which is an *exoY* mutant of ANU280 that does not synthesize any visually detectable EPS (Exo^- phenotype). The amounts of polysaccharide produced by each of these three strains at different stages of culture growth was measured using anthrone (Fig. 5.3A). Since the curves were linear, the polysaccharide values could be normalized against the protein content of the cultures (Fig. 5.3B). The quantities of polysaccharide determined by anthrone correlated with the qualitative phenotypes of the three strains on solid BMM medium. Mutant strain ANU2811 produced the least amount of hexose and since it does not synthesize any acidic EPS at all, the hexose detected by anthrone must represent the basal level due to lipopolysaccharides, endogenous sugars and possibly neutral β-1,2-glucans. Of the three strains, ANU280 produced an intermediate level of sugars detectable by anthrone. The increase above that of mutant strain ANU2811 represents the EPS synthesized by strain ANU280. Mutant strain ANU2895 produced the highest level of hexose (2.8 times that of the wild-type ANU280) and this increase reflects the level that strain ANU2895 overproduces EPS. The experiment also demonstrates that the anthrone-H_2SO_4 method was satisfactory for quantifying EPS production, above the background levels associated with non EPS sugars.

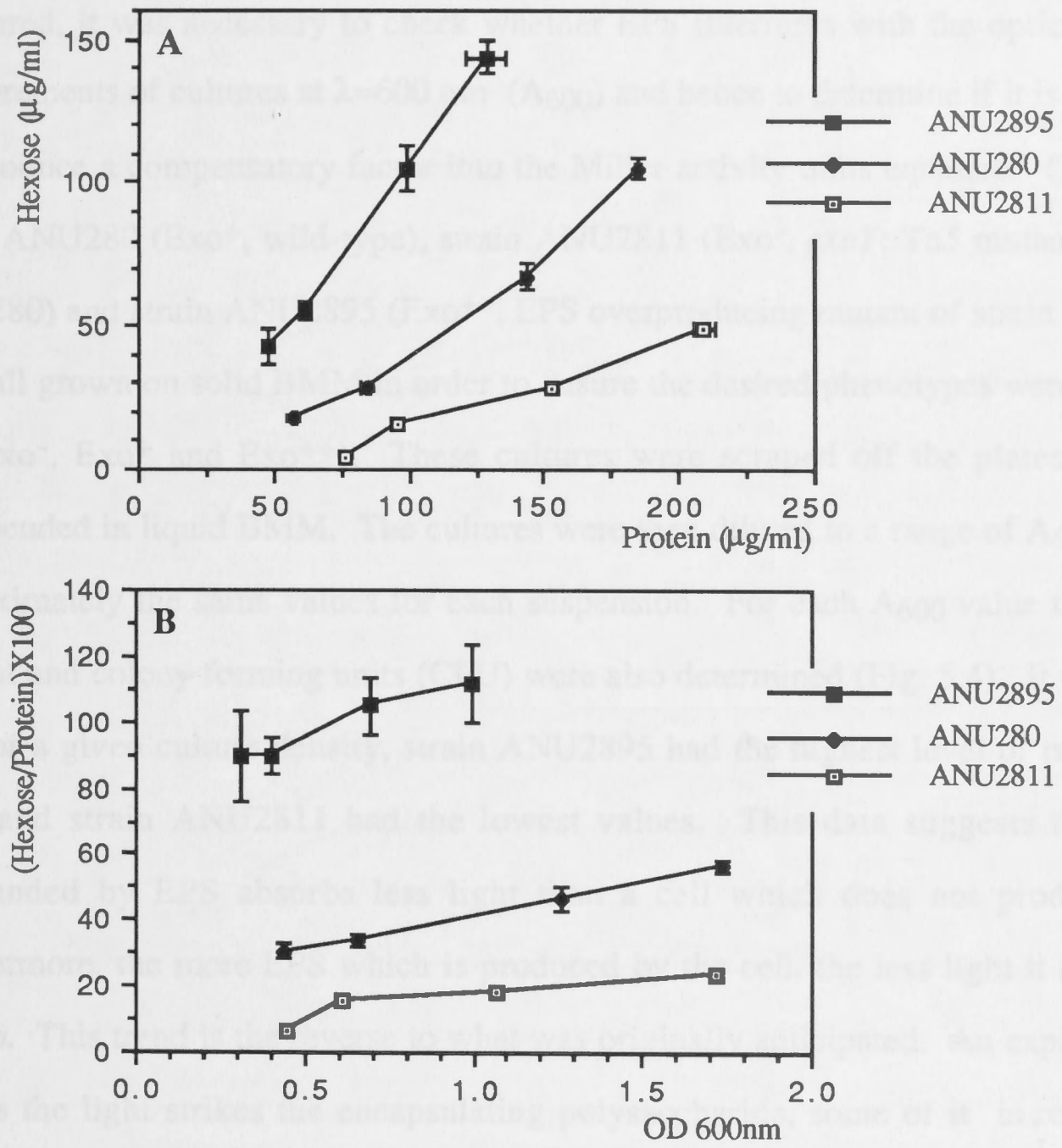


Fig. 5.3 The effectiveness of the anthrone-H_2SO_4 assay for quantifying acidic EPS. The production of of EPS by three phenotypically different *R.* sp. NGR234 strains are compared. **(A)** Compares the relative amounts of hexose accumulation in growing cultures of these three strains. **(B)** The same comparison as A, except that the hexose content has been normalized against the protein content of the culture. Strain ANU2811 was Exo^- (no EPS production), strain ANU280 was Exo^+ (wild-type) and ANU2895 was Exo^{++} (overproduction of EPS). Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

5.2.2 Interference in Optical Density Measurements caused by EPS

Since expression of *exo* promoters in backgrounds with different phenotypes were compared, it was necessary to check whether EPS interferes with the optical density measurements of cultures at λ=600 nm (A_{600}) and hence to determine if it is necessary to introduce a compensatory factor into the Miller activity units equation. Cultures of strain ANU280 (Exo^+, wild-type), strain ANU2811 (Exo^-, *exoY*::Tn*5* mutant of strain ANU280) and strain ANU2895 (Exo^{++}, EPS overproducing mutant of strain ANU280) were all grown on solid BMM in order to ensure the desired phenotypes were obtained (*ie*. Exo^-, Exo^+ and Exo^{++}). These cultures were scraped off the plates and then resuspended in liquid BMM. The cultures were then diluted to a range of A_{600} values, approximately the same values for each suspension. For each A_{600} value the protein content and colony forming units (CFU) were also determined (Fig. 5.4). It was found that for a given culture density, strain ANU2895 had the highest level of protein and CFU and strain ANU2811 had the lowest values. This data suggests that a cell surrounded by EPS absorbs less light than a cell which does not produce EPS. Furthermore, the more EPS which is produced by the cell, the less light it appears to absorb. This trend is the reverse to what was originally anticipated. An explanation is that as the light strikes the encapsulating polysaccharide, some of it is reflected or refracted rather than being absorbed. Consequently, some of the light that would normally be absorbed by a culture of Exo^- cells, was instead ricocheted through the Exo^+ and Exo^{++} cultures and still detected by the sensor of the spectrophotometer. Thus, when activities of the same promoter expressed in backgrounds of different phenotypes are to be compared, it will be necessary to introduce a multiplication factor into the equation. Based on the curves in Fig. 5.4B, these values would be 1.2 x ANU2811 = ANU280 and 0.67 x ANU2895 = ANU280. Absorbances of suspensions of the three strains at wave lengths of 420 nm and 510 nm as well as 600 nm were also measured. All three strains gave superimposable curves when plotted against their protein content, *ie*. if the three strains had equal A_{600} values, then their A_{510}

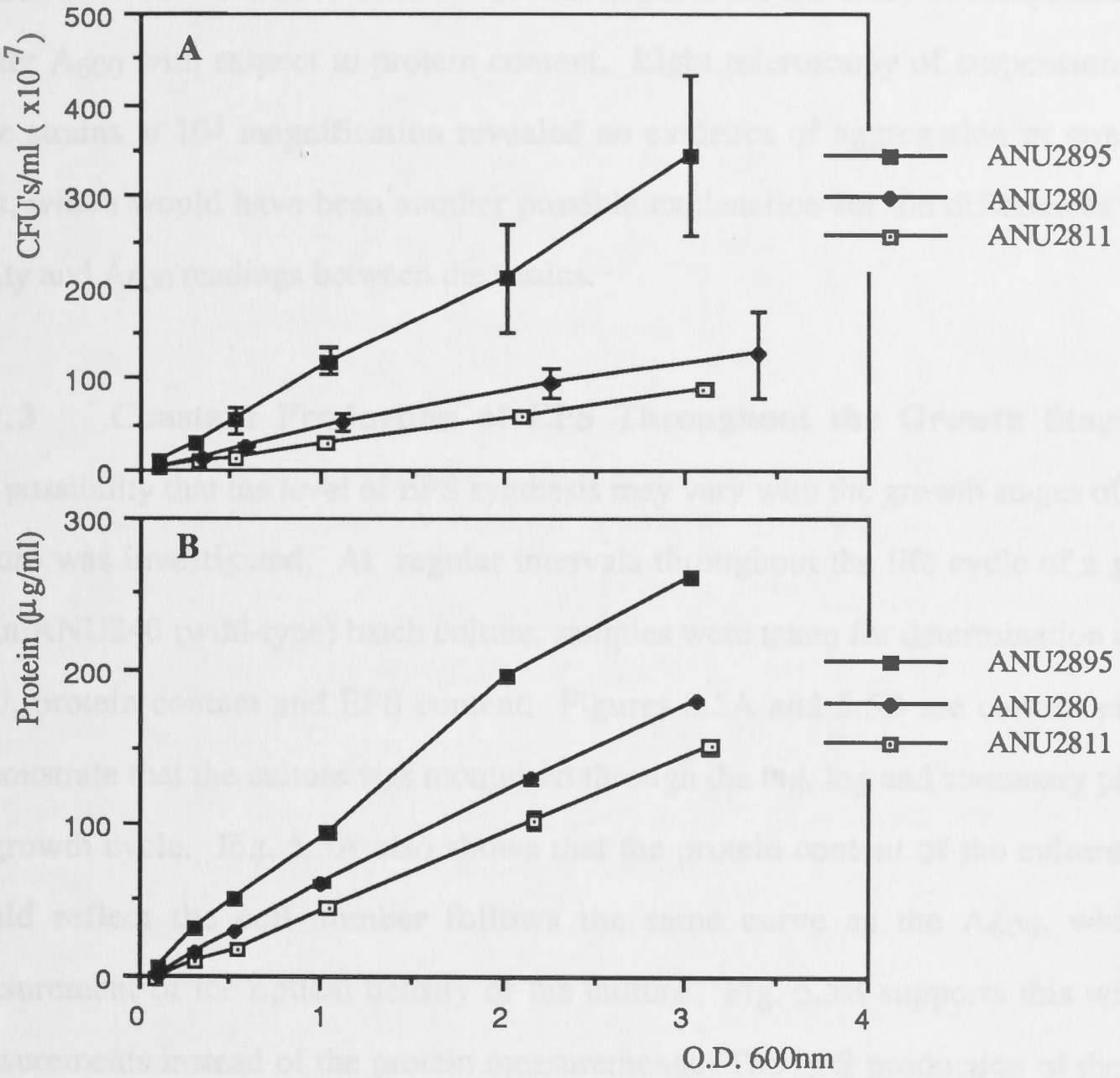


Fig. 5.4 Interference in optical density measurements caused by EPS. **(A)** Compares the concentration of colony forming units with respect to the absorbance at $\lambda = 600$ nm, between three phenotypically different *R*. sp. NGR234 strains. **(B)** Compares the protein content of the three different strains with respect to their absorbance at $\lambda = 600$ nm. Strain ANU2811 was Exo$^-$ (no EPS production), strain ANU280 was Exo$^+$ (wild-type) and strain ANU2895 was Exo^{++} (overproduction of EPS). Each plotted point represents the mean of either nine observations (A) or three observations (B). Error bars represent one standard deviation and where not visible are smaller than the symbol.

and A_{420} values were also equal. This indicates that when $\lambda = 600$ nm, proteins on the surface of strain ANU2811 cells are not excited and are therefore not responsible for a higher A_{600} with respect to protein content. Light microscopy of suspensions of the three strains at 10^3 magnification revealed no evidence of aggregation or swelling of cells, which would have been another possible explanation for the differences in CFU ability and A_{600} readings between the strains.

5.2.3 Constant Production of EPS Throughout the Growth Stages

The possibility that the level of EPS synthesis may vary with the growth stages of a batch culture was investigated. At regular intervals throughout the life cycle of a growing strain ANU240 (wild-type) batch culture, samples were taken for determination of A_{600}, CFU, protein content and EPS content. Figures 5.5A and 5.5B are control plots and demonstrate that the culture was monitored through the lag, log and stationary phases of its growth cycle. Fig. 5.5A also shows that the protein content of the culture, which would reflect the cell number follows the same curve as the A_{600}, which is a measurement of the optical density of the culture. Fig. 5.5B supports this with CFU measurements instead of the protein measurements. The EPS production of the culture during this period also follows the same curve as the A_{600} (Fig. 5.5C). This indicates that EPS production is dependent on the metabolic rates of the cell and the availability of nutrients, in the same way as protein synthesis. Polysaccharide production and protein synthesis are both linearly proportional to culture density (Fig. 5.6A). Since a linear curve and not an exponential or logarithmic curve was obtained when EPS content was plotted against culture density (Fig. 5.6A), indicates that EPS production per cell does not vary with the age of the culture. Further evidence that EPS production is constant per cell and does not vary during the growth cycle is demonstrated by the horizontally linear curve when EPS production is normalized against the protein content of the culture and plotted against cell density (Fig. 5.6B). The absence of any slope is due to the absence

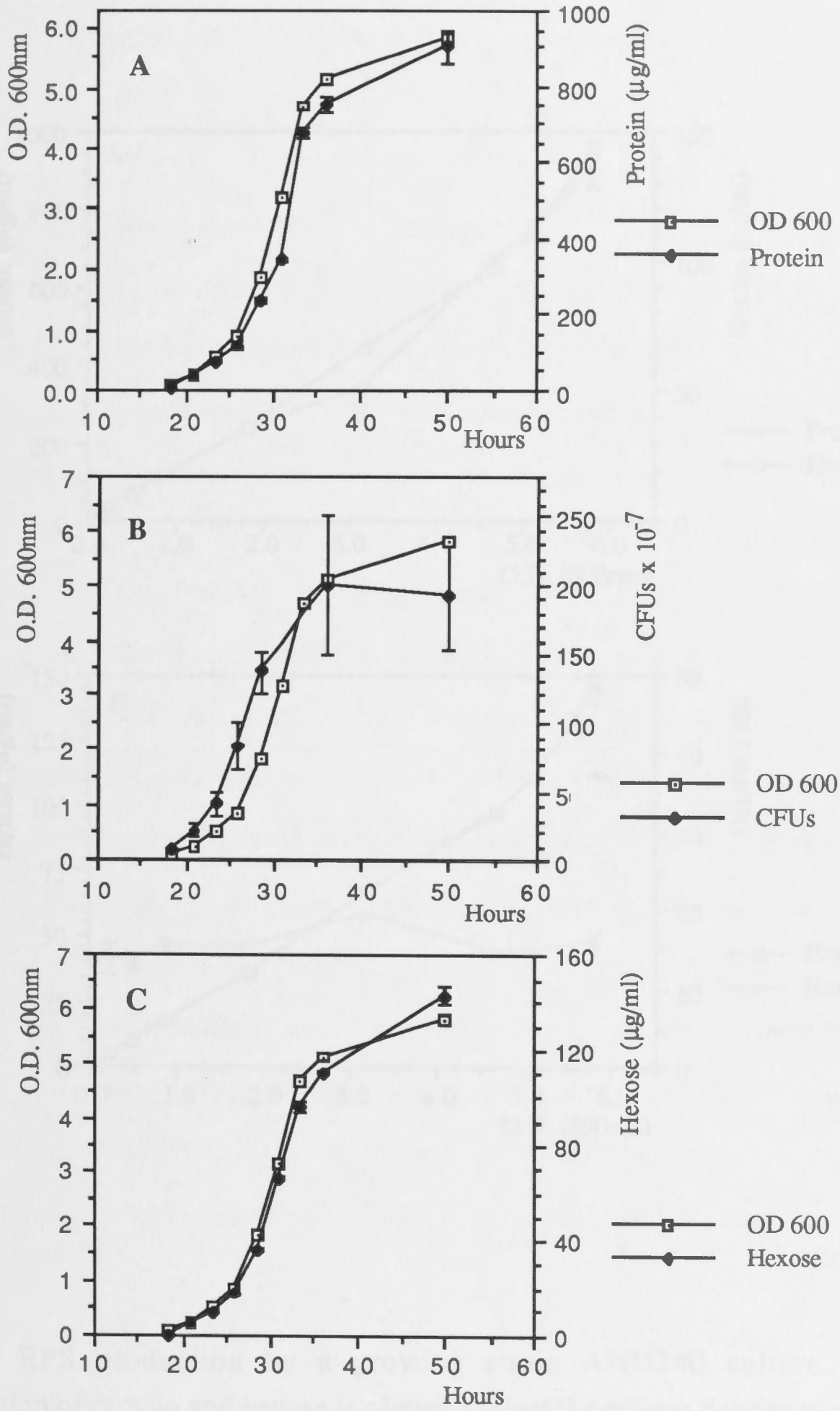


Fig. 5.5 Measurements of protein, CFUs and hexose from a growing wild-type strain ANU240 culture. Culture growth is represented by the increasing optical density measurements at $\lambda = 600$ nm. **(A)** Accumulation of protein within a growing batch culture. **(B)** Accumulation of CFUs within a growing batch culture. **(C)** Accumulation of hexose within a growing batch culture. Each plotted point represents the mean of at least three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

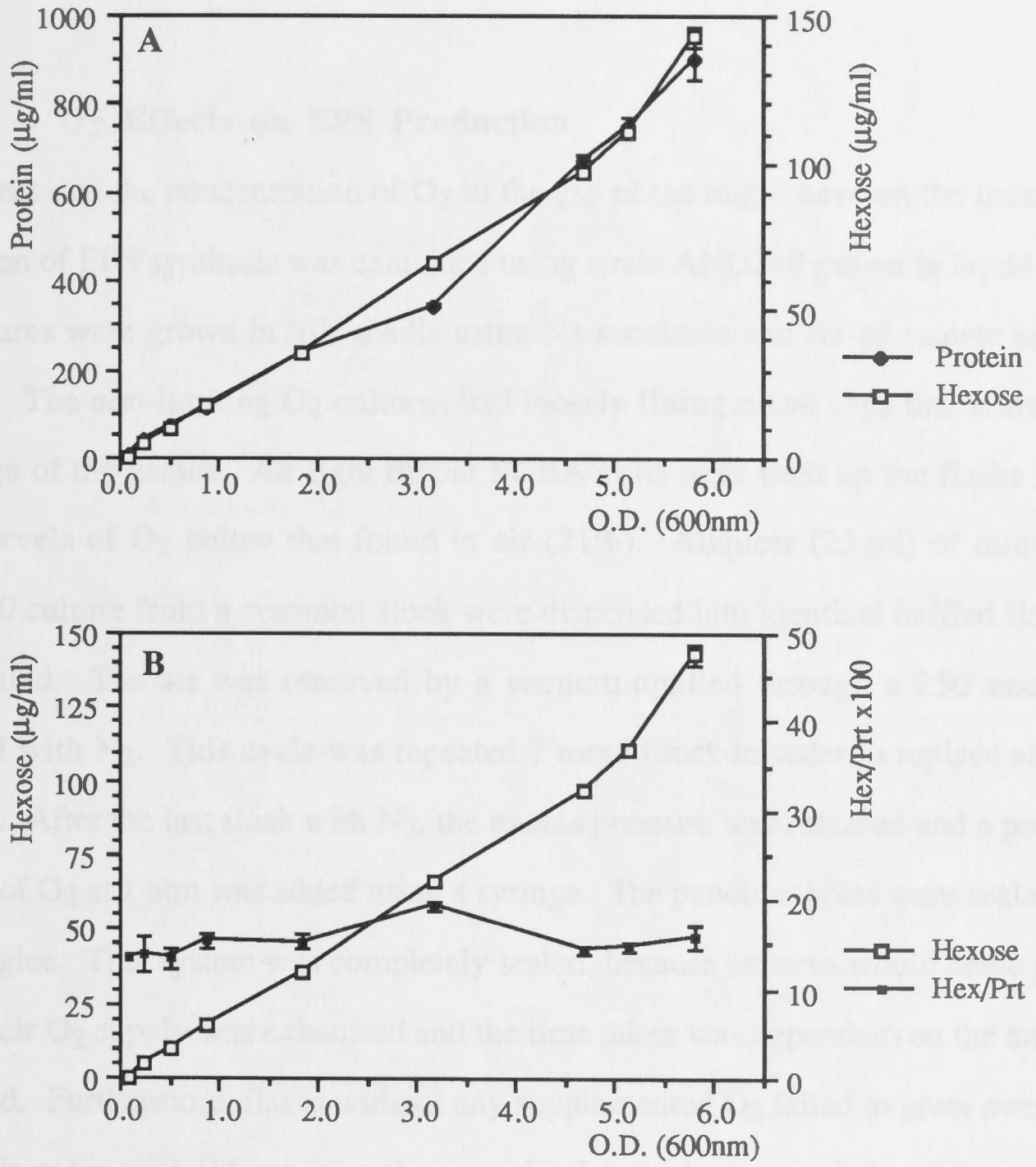


Fig. 5.6 EPS production by a growing strain ANU240 culture. **(A)** The accumulation of protein and hexose is plotted against the culture density as measured by absorbance at $\lambda = 600$ nm. **(B)** Hexose accumulation when normalized against protein accumulation is plotted against culture density and compared with hexose accumulation. Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

of any trend for the rate of EPS production to either increase or decrease as the culture ages.

5.2.4 O_2 Effects on EPS Production

Any effect that the concentration of O_2 in the gas phase might have on the induction or repression of EPS synthesis was examined using strain ANU240 grown in liquid culture. All cultures were grown in MX media using Na-succinate and Na-glutamate as carbon sources. The non-limiting O_2 cultures had loosely fitting metal caps that allowed free exchange of the gasses. Air tight rubber SUBA seals were used on the flasks that had varied levels of O_2 below that found in air (21%). Aliquots (25 ml) of dilute strain ANU240 culture from a common stock were dispensed into identical baffled flasks and then sealed. The air was removed by a vacuum applied through a 25G needle and replaced with N_2. This cycle was repeated 7 more times in order to replace all the air with N_2. After the last flush with N_2, the excess pressure was released and a prescribed volume of O_2 at 1 atm was added using a syringe. The puncture holes were sealed with a drop of glue. This system was completely sealed, because cultures would cease growing when their O_2 supply was exhausted and the time taken was dependent on the amount of O_2 added. Furthermore, flasks without any supplemented O_2 failed to grow even after 1 week. In order to avoid arresting the growth of the culture, every 6 to 8 hours the gas volume of the flasks was again flushed with N_2 and the desired O_2 conditions renewed.

The amount of EPS produced by a growing strain ANU240 culture was inversely proportional to the amount of O_2 available, *ie*. as the O_2% in the gas phase decreased, more EPS was produced per cell (Fig. 5.7A). This is misleading, because the culture densities and times taken to reach those densities (Fig.5.7A) indicate that the cell replication time was also inversely proportional to the O_2 availability. In this experiment the nutrients of the media are in abundant supply and the only limitation is the O_2 availability. Therefore, despite the dramatic decrease in the rates of protein synthesis and

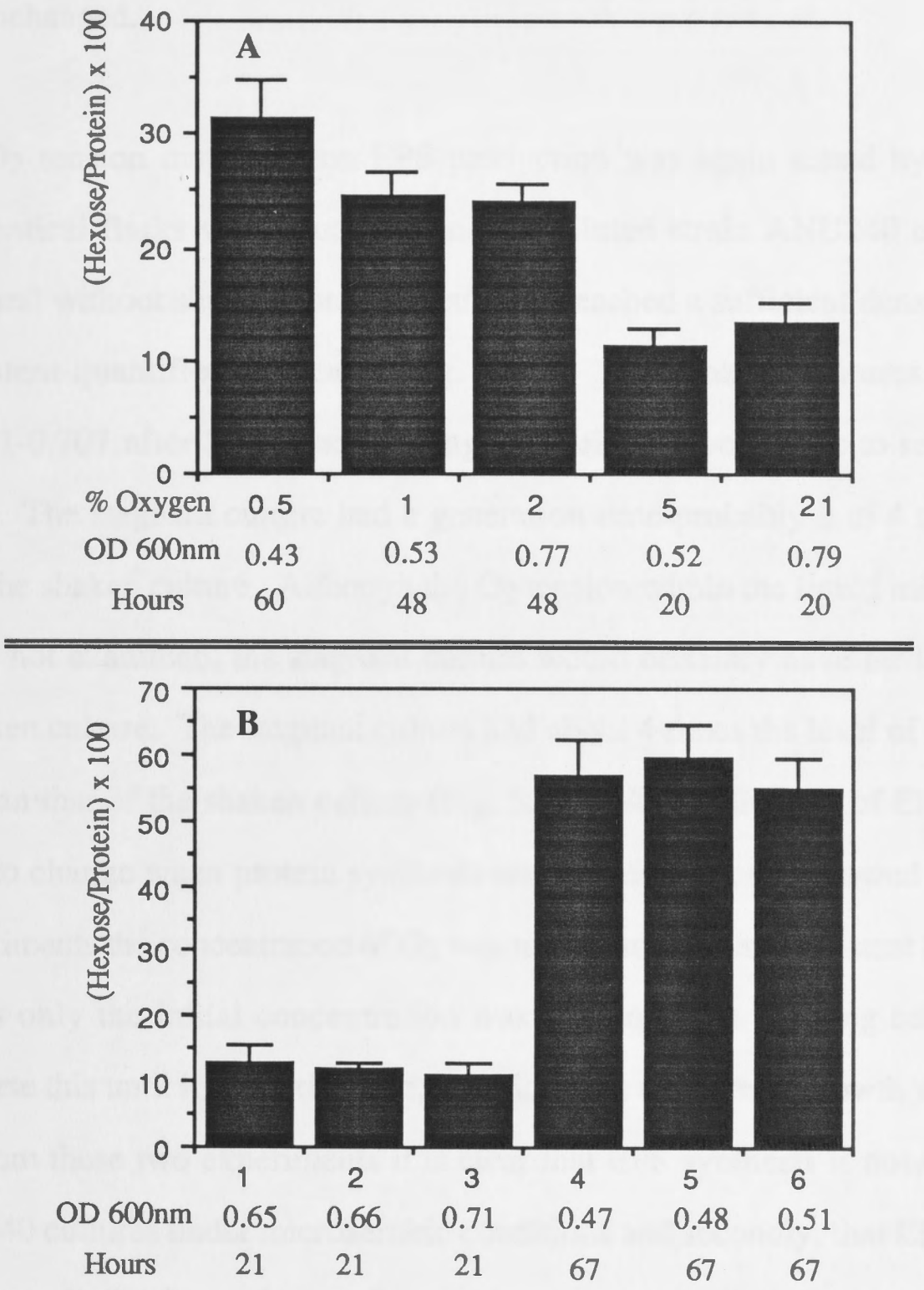


Fig. 5.7 O_2 effects on EPS production of strain ANU240. **(A)** Hexose accumulation normalized against protein accumulation in cultures grown in a range of O_2 concentrations in the gas phase. **(B)** Hexose accumulation normalized against protein accumulation in cultures grown under different conditions. Samples 1, 2 and 3 were grown with vigorous shaking to promote maximum aeration and cultures 4, 5 and 6 were grown without shaking. The culture densities (A_{600}) and incubation period for each sample is indicated. Each plot represents the mean of either 15 observations (A) or three observations (B). Error bars represent one standard deviation.

cell replication under these conditions, the rate (per unit time) of EPS production remains essentially unchanged.

The effect O_2 tension may have on EPS production was again tested by a different method. Identical flasks with equal volumes of diluted strain ANU240 culture were grown with and without shaking, until the cultures reached a sufficient density and then the EPS content quantified for each (Fig. 5.7B). The shaking cultures reached an A_{600} = 0.651-0.707 after 21 hr and the stagnant cultures took 67 hr to reach A_{600} = 0.471-0.513. The stagnant culture had a generation time probably 3 to 4 times longer than that of the shaken culture. Although the O_2 tension within the liquid medium of the cultures was not examined, the stagnant culture would certainly have far less aeration than the shaken culture. The stagnant culture had about 4 times the level of EPS per mg of protein than that of the shaken culture (Fig. 5.7B). Again, the rate of EPS synthesis appears not to change when protein synthesis and cell division was slowed right down. In both experiments the concentration of O_2 was not maintained at a constant level. In the sealed flasks only the initial concentration was known and a growing culture would steadily deplete this until it was exhausted, at which time the culture growth was arrested. However, from these two experiments it is clear that EPS synthesis is not repressed in strain ANU240 cultures under microaerobic conditions and secondly, that EPS synthesis is not linked to cell division under conditions where nutrients are not limiting.

5.2.5 Response of *exoX* and *exoY* Promoters to low O_2

The β-galactosidase activities associated with four fusion clones were examined under microaerobic conditions in the wild-type strain ANU240 background. The plasmid constructs were: pJG54 (*exoY'-lacZ*$^+$), pJG66 (ORF1'-*lacZ*$^+$), pJG60 (*exoX'-lacZ*$^+$), pARI7 (putative 2895'-*lacZ*$^+$) (Figs. 5.1 and 5.2). The strains carrying the constructs were cultured to mid log phase in liquid MX media with 20 mM succinate and 20 mM L-arabinose as carbon sources. The cultures were then dispensed as 20 ml aliquots into

baffled flasks, sealed with SUBA stoppers and the gas phase replaced with N_2 and O_2 with varying degrees of microaerobicity as described in section 5.2.4. The concentration of β-galactosidase was assayed after induction periods of either 4 hr or 24 hr. There was no difference in the expression of the *lacZ* gene with either of the induction periods, so only the 4 hr induction results are presented (Fig. 5.8). Microaerobic conditions, including zero O_2, had no effect on any of the promoters. The fact that as much cell division occurred in the samples with an initial 5% O_2 concentration as occurred under non-limiting O_2 conditions (Fig. 5.8), indicates that these cells (5% O_2) had ample O_2 to sustain all of their metabolic respiratory demands for the 4 hr. The samples with zero O_2 showed no further cell division. Therefore, this range of O_2 concentrations should have been adequate to detect any regulatory effects caused by microaerobic conditions.

5.2.6 Decrease in Copy number of IncP1 Plasmids in the Absence of Selection

Liquid cultures of transconjugants were grown in the absence and in the presence of tetracycline at 0.4 $\mu g.ml^{-1}$ and their abilities to retain plasmids were demonstrated by contrasting the levels of β-galactosidase expression. The results for four plasmids carried in wild-type strains are presented in Fig. 5.9. The rapid logarithmic decrease in β-galactosidase activity that occurs when cultures are grown in the absence of tetracycline, represents a steady decrease in copy number of the plasmid per cell. The approximately constant β-galactosidase activity through out the growth of a culture grown in the presence of 0.4 $\mu g.ml^{-1}$ of tetracycline, indicates that the copy number of the plasmid is not changing. Therefore, when comparing activities of cultures at different stages of growth, it was necessary to include tetracycline at 0.4 $\mu g.ml^{-1}$. Tetracycline at this concentration did not noticeably increase or decrease the cell replication rate of the culture.

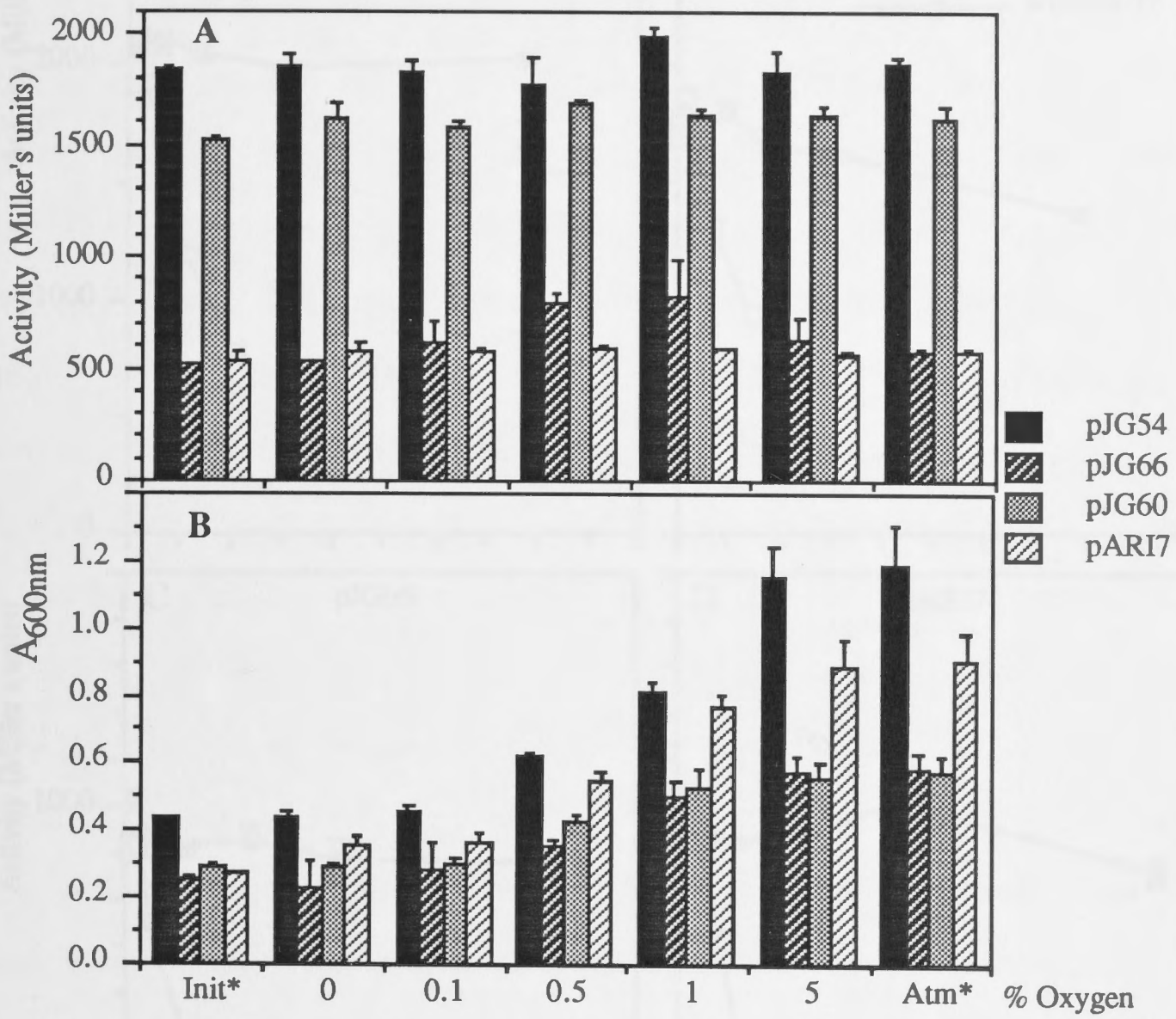


Fig. 5.8 **(A)** Transcription of *exo* promoters in strain ANU240 at a range of O_2 concentrations in the gas phase. Expression was determined by measuring β-galactosidase activity (Miller, 1972) of a transcriptional *lacZ* gene fusion to either *exoY* (pJG54), ORF1 (pJG66), *exoX* (pJG60) or the putative 2895 gene (pARI7). **(B)** Growth of the cultures assayed in (A) after a 4 hr incubation period at a range of O_2 concentrations in the gas phase. Each plot represents the mean of 12 observations. Error bars represent one standard deviation.

* Init: Analysis of initial culture prior to induction period,
Atm: Free gas exchange between flask and atmosphere.

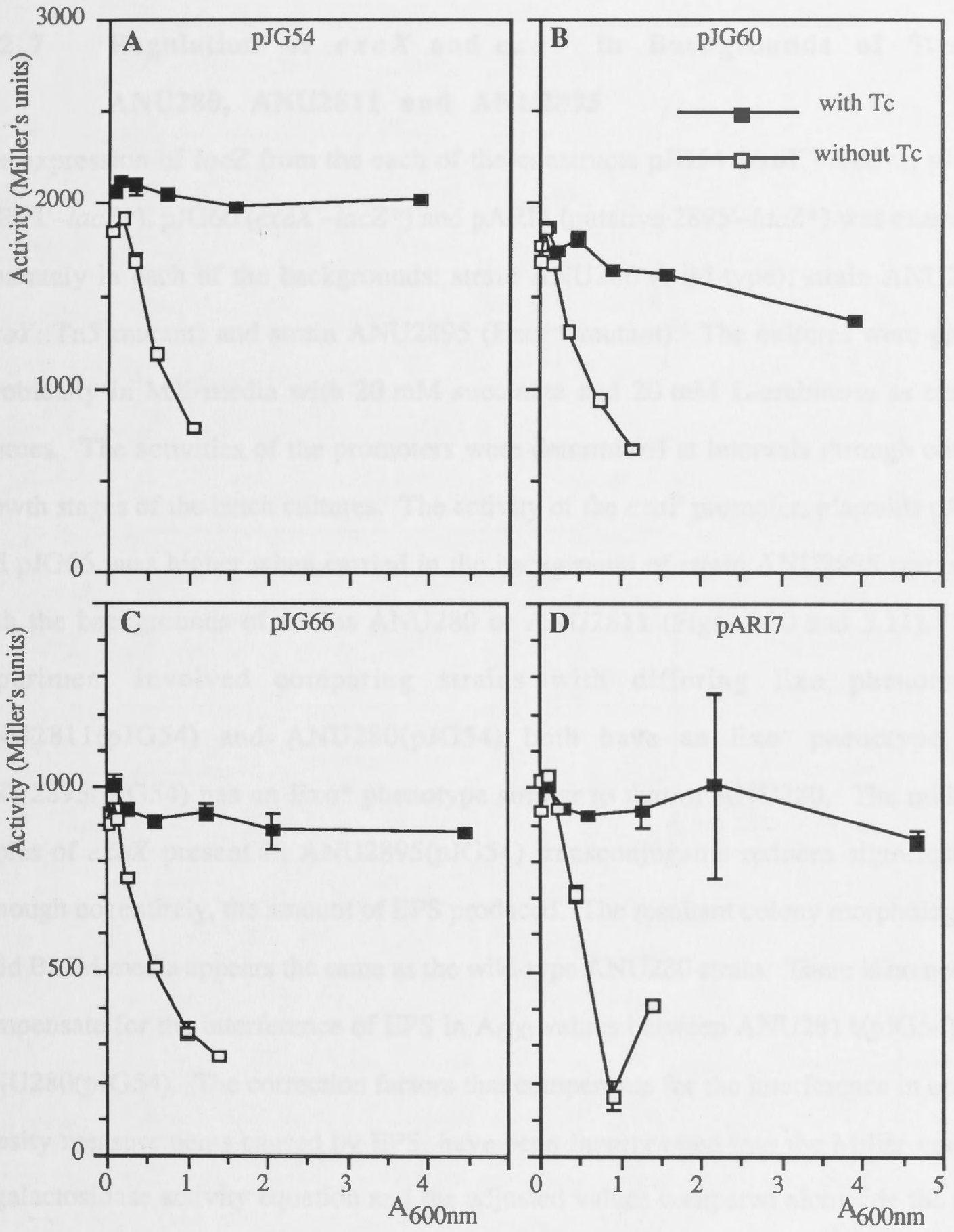


Fig. 5.9 A comparison of β-galactosidase activity of transcriptional *lacZ* gene fusion constructs in strain ANU240 when cultured in the presence or absence of tetracycline (see legend in frame B). The activity of β-galactosidase was normalized against cell density as defined by Miller (1972). **(A)** pJG54 (*exoY'-lacZ*$^+$), **(B)** pJG60 (*exoX'-lacZ*$^+$), **(C)** pJG66 (ORF1'-*lacZ*$^+$), **(D)** pARI7 (putative 2895'-*lacZ*$^+$). Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

5.2.7 Regulation of *exoX* and *exoY* in Backgrounds of Strains ANU280, ANU2811 and ANU2895

The expression of *lacZ* from the each of the constructs pJG54 (*exoY'–lacZ*$^+$), pJG66 (ORF1'–*lacZ*$^+$), pJG60 (*exoX'–lacZ*$^+$) and pARI7 (putative 2895'–*lacZ*$^+$) was examined separately in each of the backgrounds: strain ANU280 (wild-type), strain ANU2811 (*exoY*::Tn*5* mutant) and strain ANU2895 (Exo^{++} mutant). The cultures were grown aerobically in MX media with 20 mM succinate and 20 mM L-arabinose as carbon sources. The activities of the promoters were determined at intervals through out the growth stages of the batch cultures. The activity of the *exoY* promoter, plasmids pJG54 and pJG66, was higher when carried in the background of strain ANU2895 compared with the backgrounds of strains ANU280 or ANU2811 (Figs. 5.10 and 5.11). This experiment involved comparing strains with differing Exo phenotypes: ANU2811(pJG54) and ANU280(pJG54) both have an Exo$^-$ phenotype, but ANU2895(pJG54) has an Exo$^+$ phenotype similar to that of ANU280. The multiple copies of *exoX* present in ANU2895(pJG54) transconjugants reduces significantly, although not entirely, the amount of EPS produced. The resultant colony morphology on solid BMM media appears the same as the wild-type ANU280 strain. There is no need to compensate for the interference of EPS in A_{600} values between ANU2811(pJG54) and ANU280(pJG54). The correction factors that compensate for the interference in optical density measurements caused by EPS, have been incorporated into the Miller units of β-galactosidase activity equation and the adjusted values compared alongside the non-adjusted values for all promoters examined (Figs. 5.10, 5.11, 5.12 and 5.13). The activities of the *exoX* (pJG60) and the 2895 (pARI7) promoters were only slightly affected in the different genetic backgrounds (Figs. 5.12 and 5.13). The differences were relatively minor that it is unlikely that *exoX* or 2895 promoters are transcriptionally regulated by *exoY* or 2895 gene products. However, the expression of the *exoY*-ORF1 promoter (from pJG54 or pJG66) was 2.5 to 5 fold higher when in the mutant

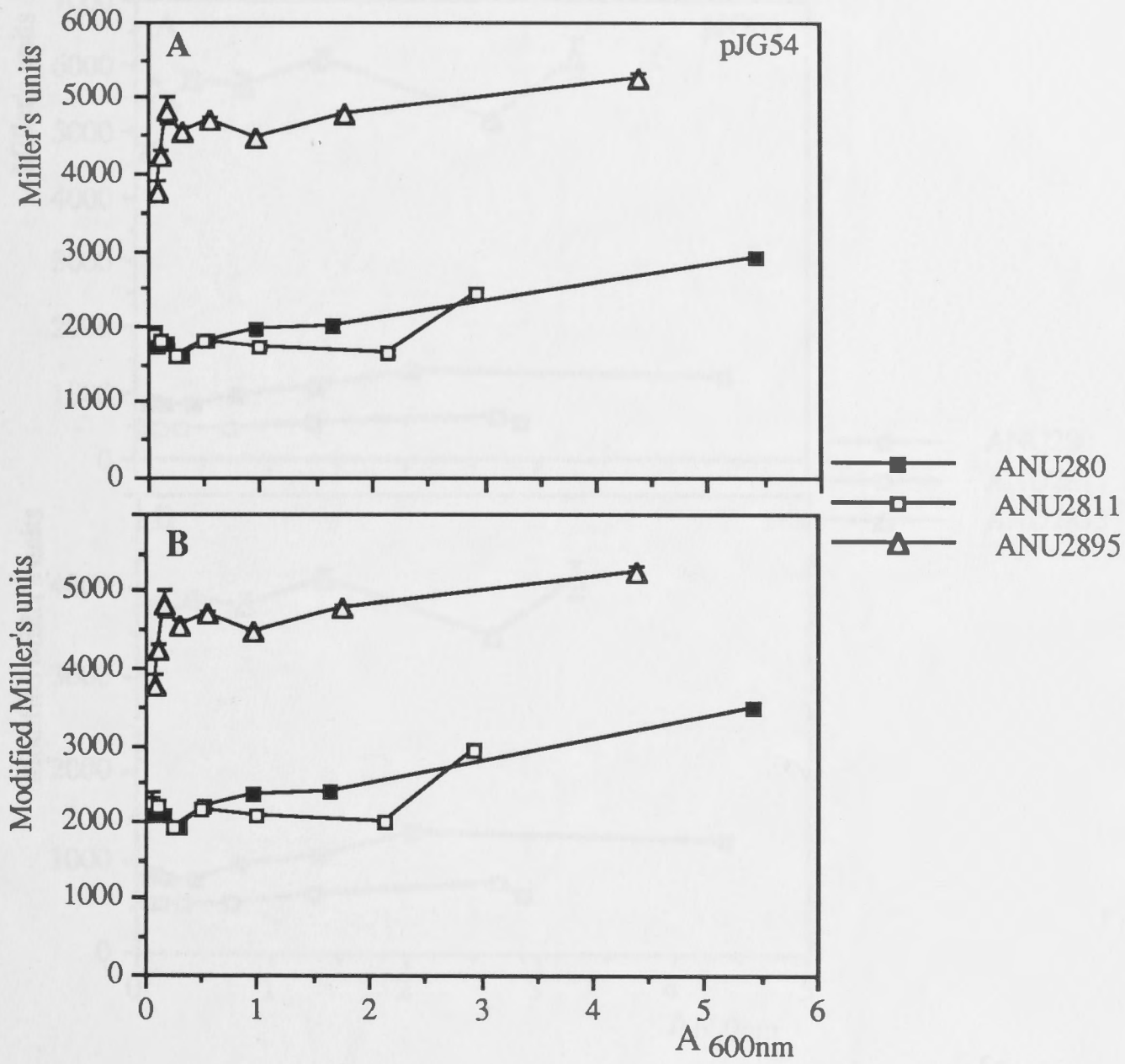


Fig. 5.10 Regulation of the *exoY* promoter in growing backgrounds of strains ANU280 (wild-type), ANU2811 (*exoY*::Tn*5*) and ANU2895 (2895::Tn5). The plasmid construct used was pJG54, which is a transcriptional fusion *exoY'-lacZ*$^+$. **(A)** β-galactosidase activity is represented in the units as defined by Miller (1972). **(B)** The β-galactosidase activity measurements of (A) have been modified using the correction factors determined in section 5.2.2. Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

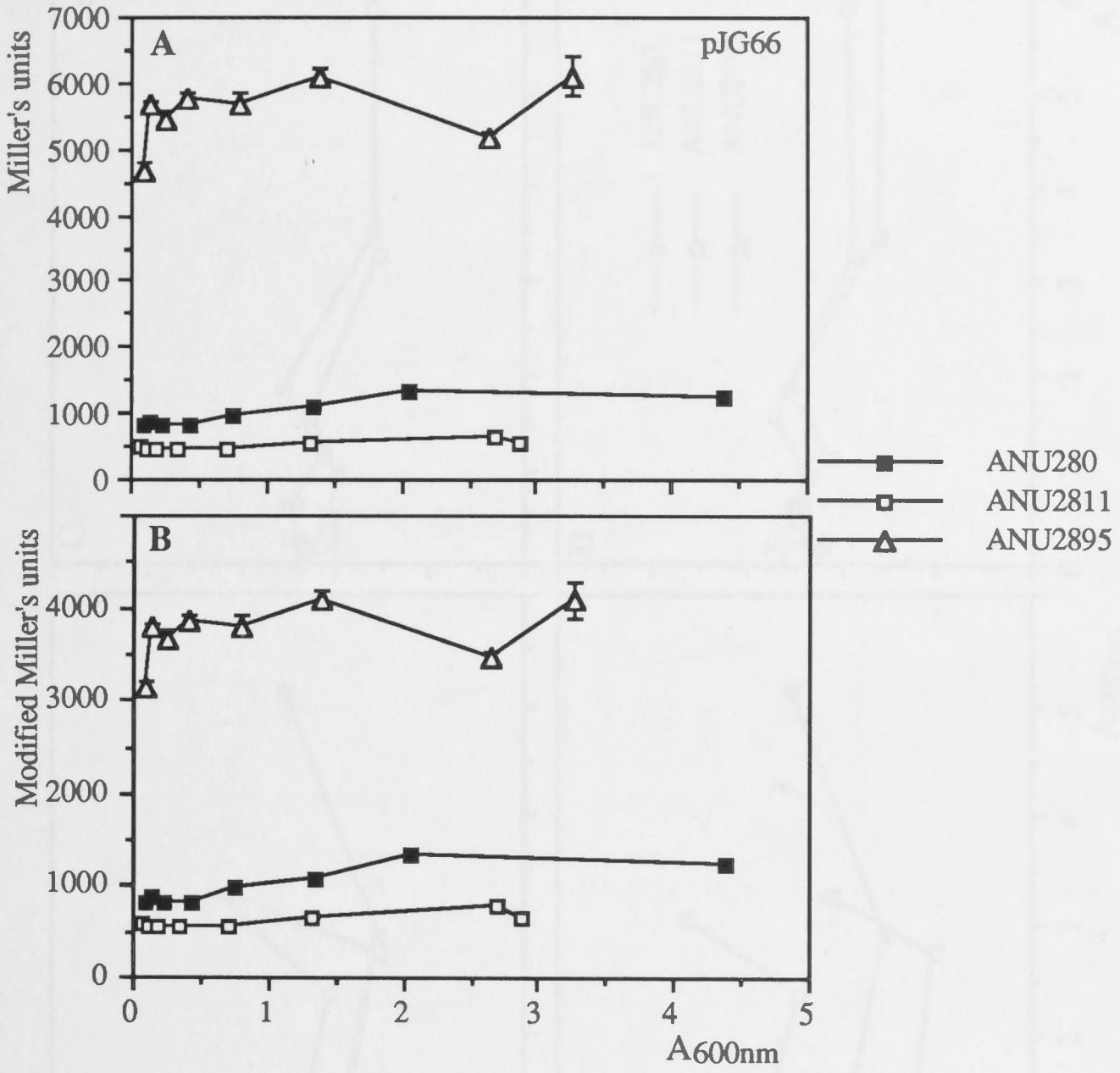


Fig. 5.11 Regulation of the *exoY*-ORF1 promoter in growing backgrounds of strains ANU280 (wild-type), ANU2811 (*exoY*::Tn*5*) and ANU2895 (2895::Tn5). The plasmid construct used was pJG66, which is a transcriptional fusion ORF1'-*lacZ*$^+$. **(A)** β-galactosidase activity is represented in the units as defined by Miller (1972). **(B)** The β-galactosidase activity measurements of (A) have been modified using the correction factors determined in section 5.2.2. Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

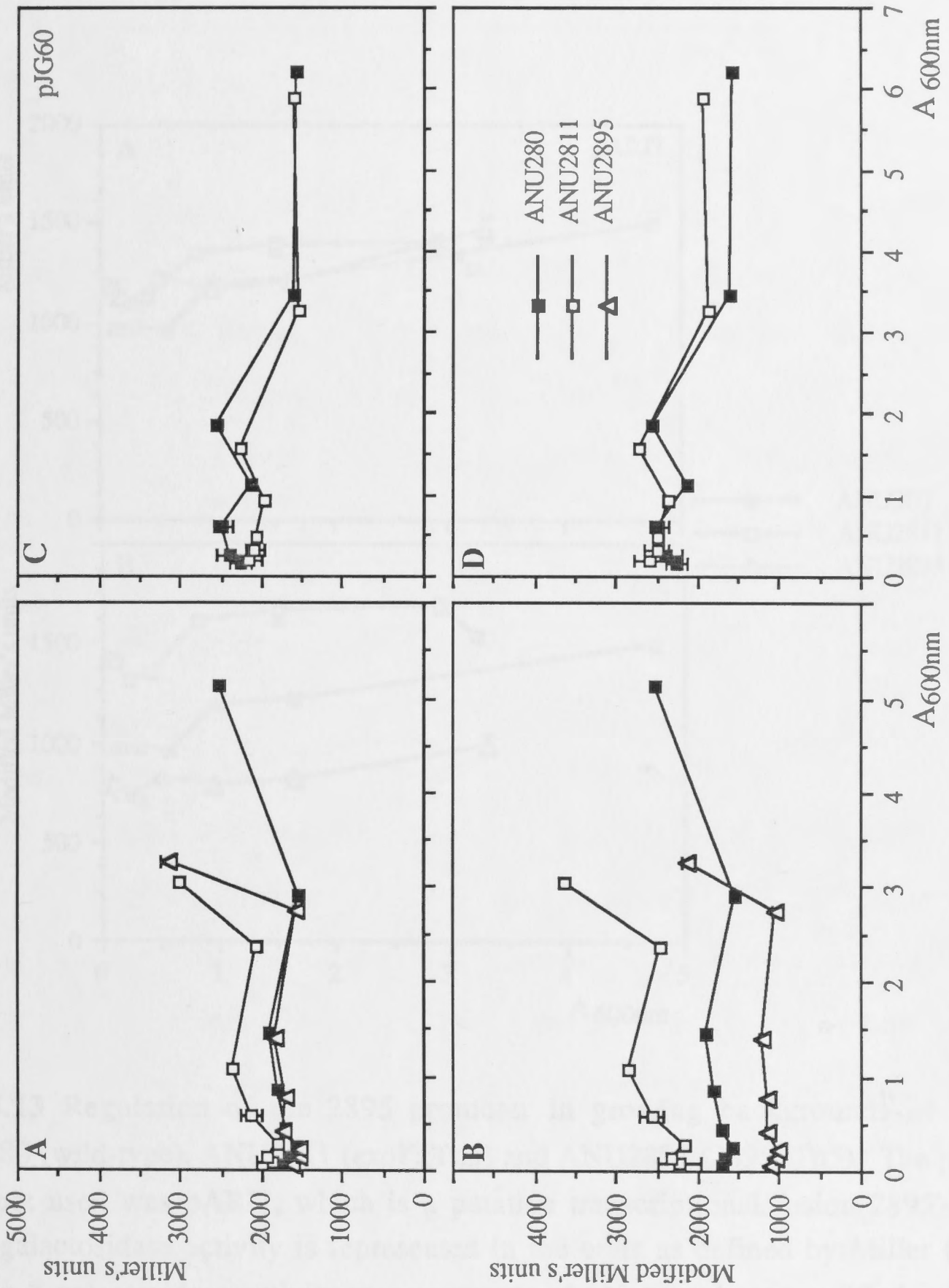


Fig. 5.12 Regulation of the *exoX* promoter in growing backgrounds of strains ANU280 (wild-type), ANU2811 (*exoY*::Tn*5*) and ANU2895 (2895::Tn5). The plasmid construct used was pJG60, which is a transcriptional fusion *exoX'–lacZ*$^+$. **(A)** β-galactosidase activity is represented in the units as defined by Miller (1972). **(B)** The β-galactosidase activity measurements of (A) have been modified using the correction factors determined in section 5.2.2. **(C)** and **(D)** are repeat experiments of (A) and (B) respectively in the backgrounds of strains ANU280 and ANU2811. Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol. The legend of plot symbols for all graphs is shown in frame (D).

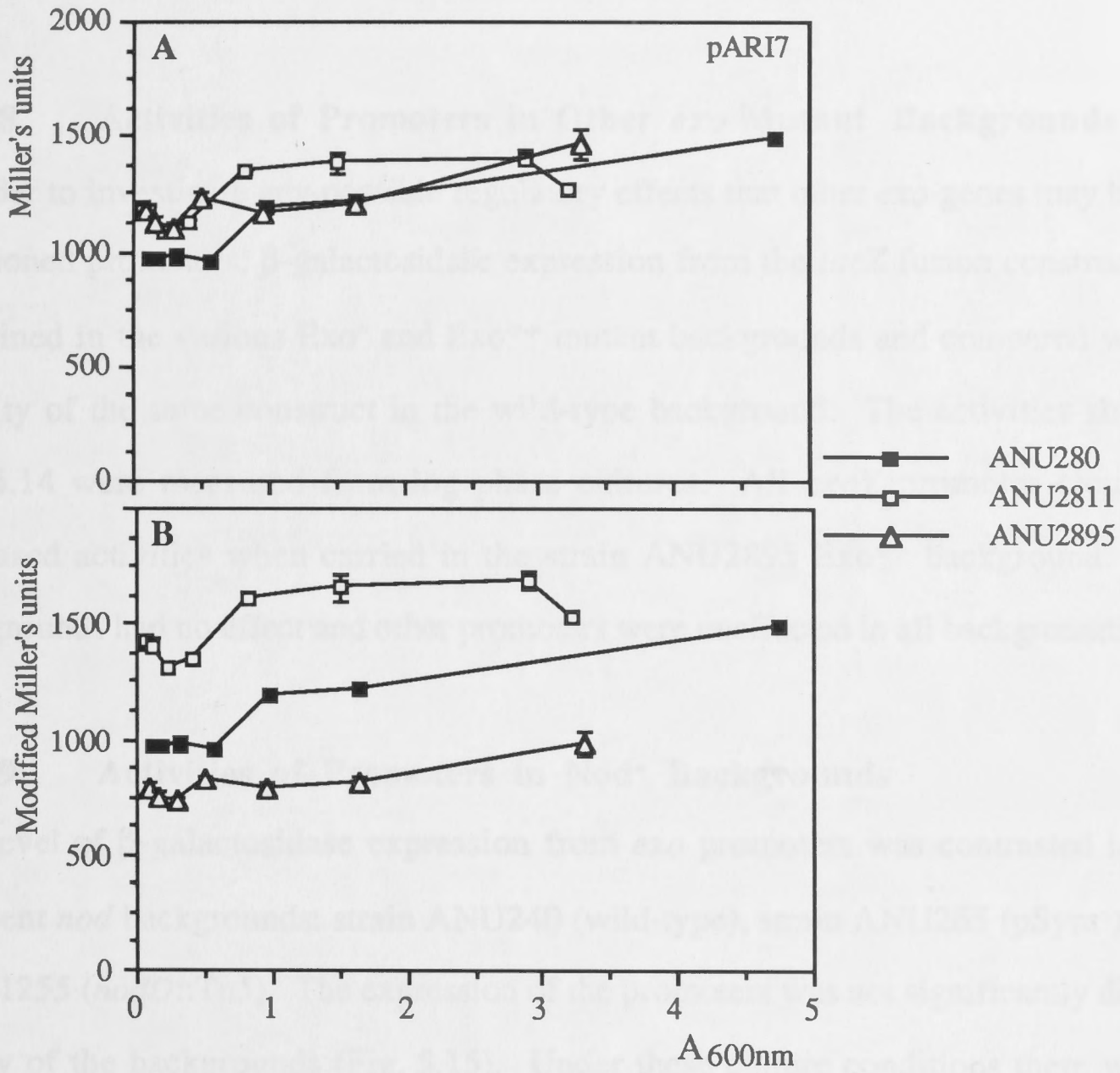


Fig. 5.13 Regulation of the 2895 promoter in growing backgrounds of strains ANU280 (wild-type), ANU2811 (*exoY*::Tn*5*) and ANU2895 (2895::Tn5). The plasmid construct used was pARI7, which is a putative transcriptional fusion 2895'-*lacZ*$^+$. **(A)** β-galactosidase activity is represented in the units as defined by Miller (1972). **(B)** The β-galactosidase activity measurements of (A) have been modified using the correction factors determined in section 5.2.2. Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

background of strain ANU2895 compared to the backgrounds of strains ANU280 or ANU2811 (Figs. 5.10 and 5.11).

5.2.8 Activities of Promoters in Other *exo* Mutant Backgrounds

In order to investigate any possible regulatory effects that other *exo* genes may have on the cloned promoters, β-galactosidase expression from the *lacZ* fusion constructs was examined in the various Exo^- and Exo^{++} mutant backgrounds and compared with the activity of the same construct in the wild-type background. The activities shown in Fig. 5.14 were measured from log phase cultures. All *exoY* promoter clones had increased activities when carried in the strain ANU2895 Exo^{++} background. Other backgrounds had no effect and other promoters were unaffected in all backgrounds.

5.2.9 Activities of Promoters in Nod^- Backgrounds

The level of β-galactosidase expression from *exo* promoters was contrasted in three different *nod* backgrounds: strain ANU240 (wild-type), strain ANU265 ($pSym^-$), strain ANU1255 (*nodD*::Tn*5*). The expression of the promoters was not significantly different in any of the backgrounds (Fig. 5.15). Under these culture conditions there were no genetic elements on the symbiotic plasmid including *nodD*, that influenced the expression of the cloned *exo* promoters examined. This is not surprising, since there are also no detectable changes in the Exo phenotype of the strains ANU265 and ANU1255 compared to strain ANU240.

5.2.10 Regulation of *exoX* and *exoY* Promoters by Different Carbon Sources

Possible regulatory effects that the carbon source may have on the activities of *exo* promoters was investigated by measuring *lacZ* expression of transconjugant cultures grown in a range of different carbon sources at 40 mM. The wild-type strain produces EPS, albeit varying amounts, on all carbon sources examined. The following carbon

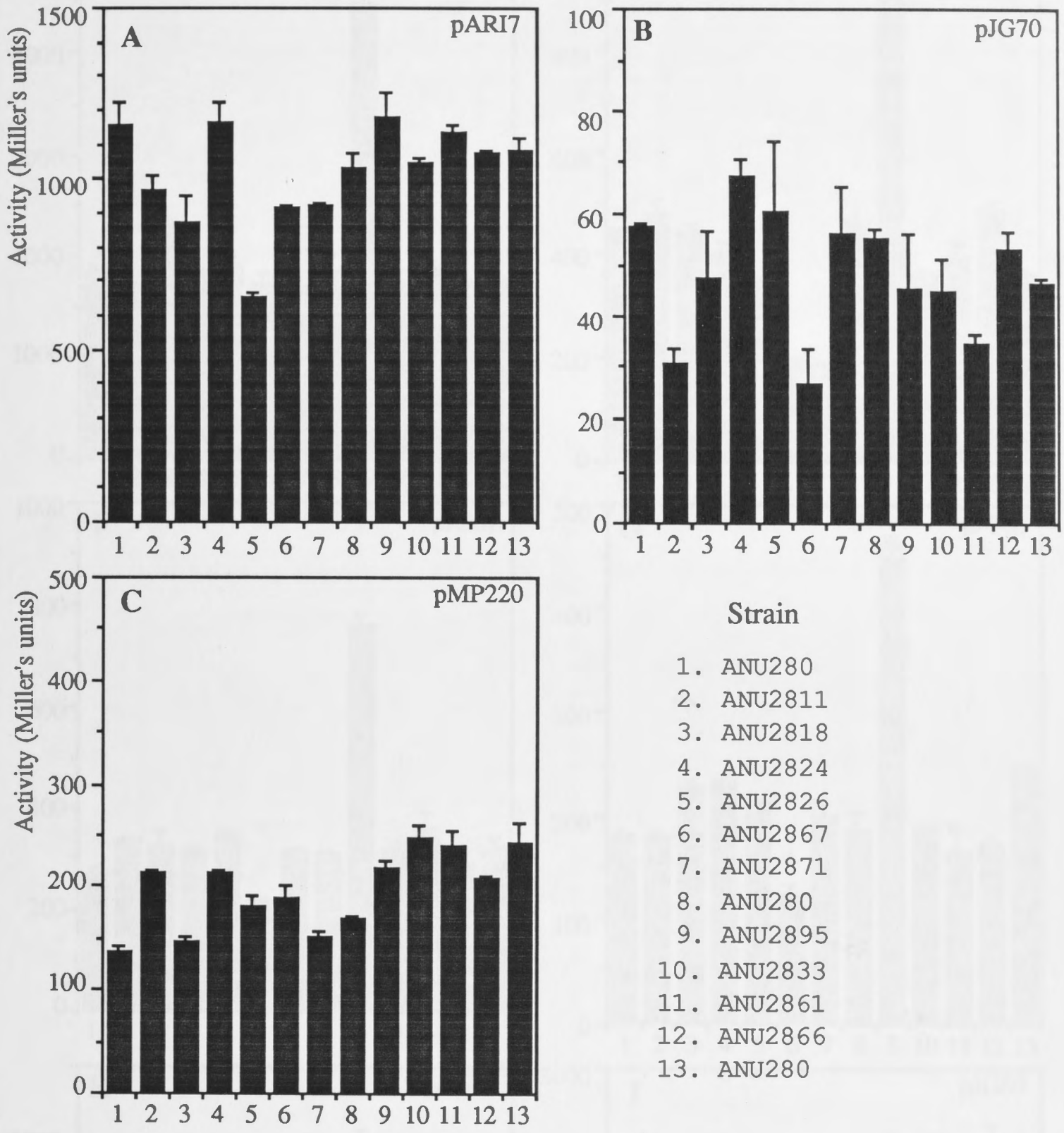
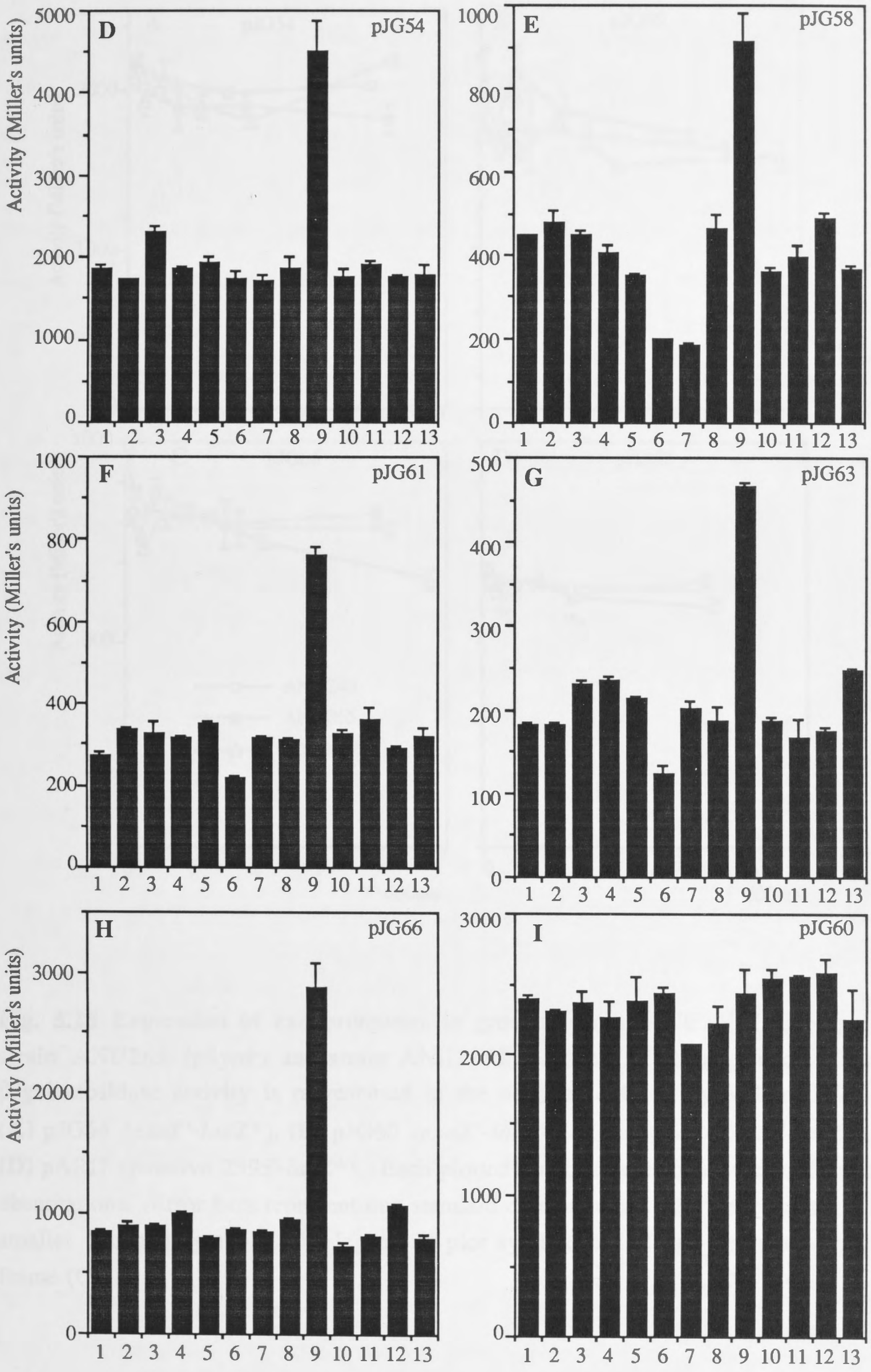


Fig. 5.14 Expression of promoters in various *exo* mutant backgrounds. β-galactosidase activity is represented in the units as defined by Miller (1972). **(A)** pARI7 (putative 2895'-*lacZ*$^+$), **(B)** pJG70 (transcriptional terminator-*lacZ*$^+$, **(C)** pMP220 (vector), **(D)** pJG54 (*exoY'-lacZ*$^+$), **(E)** pJG58 (*exoY'-lacZ*$^+$), **(F)** pJG61 (*exoY'-lacZ*$^+$), **(G)** pJG63 (*exoY'-lacZ*$^+$), **(H)** pJG66 (ORF1'-*lacZ*$^+$), **(I)** pJG60 (*exoX'-lacZ*$^+$). The lengths of DNA involved in each fusion construct is shown in figures 5.1 and 5.2. Each plot represents the mean of three observations. Error bars represent one standard deviation.


D
pJG54
E
pJG58
F
pJG61
G
pJG63
H
pJG66
I
pJG60
Activity (Miller's units)
0
200
400
600
800
1000
1200
1400
1600
1800
2000
3000
4000
5000
100
300
500
1 2 3 4 5 6 7 8 9 10 11 12 13

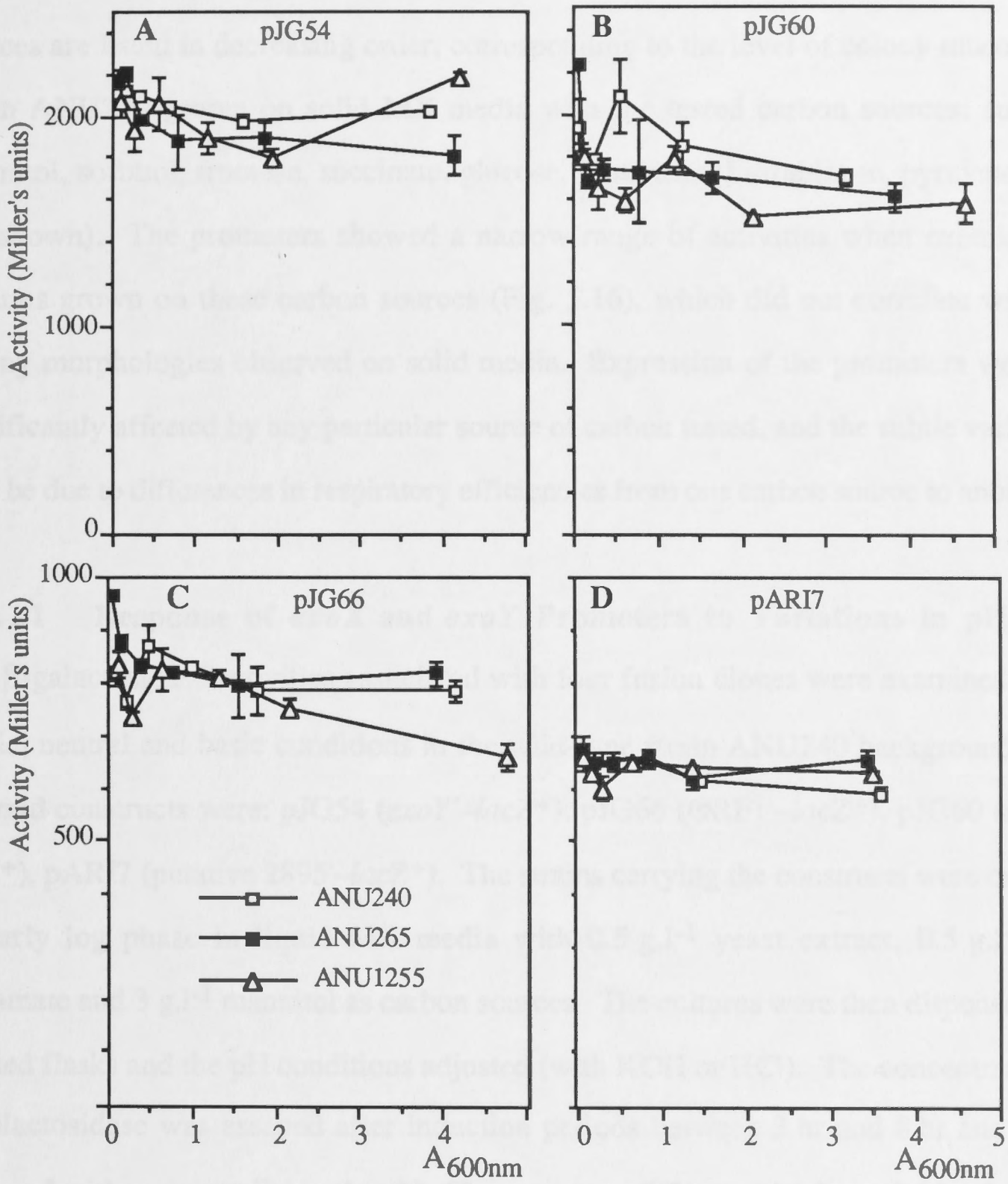


Fig. 5.15 Expression of *exo* promoters in growing strain ANU240 (wild-type), strain ANU265 (pSym$^-$) and strain ANU1255 (*nodD*::Tn*5*) backgrounds. The β-galactosidase activity is represented in the units as defined by Miller (1972). **(A)** pJG54 (*exoY'-lacZ*$^+$), **(B)** pJG60 (*exoX'-lacZ*$^+$), **(C)** pJG66 (ORF1'-*lacZ*$^+$), **(D)** pARI7 (putative 2895'-*lacZ*$^+$). Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol. The legend of plot symbols for all graphs is shown in frame (C).

sources are listed in decreasing order, corresponding to the level of colony mucoidy of strain ANU240 grown on solid MX media with the tested carbon sources: sucrose, mannitol, sorbitol, fructose, succinate, glucose, glutamate, L-arabinose, pyruvate (data not shown). The promoters showed a narrow range of activities when examined in cultures grown on these carbon sources (Fig. 5.16), which did not correlate with the colony morphologies observed on solid media. Expression of the promoters were not significantly affected by any particular source of carbon tested, and the subtle variations may be due to differences in respiratory efficiencies from one carbon source to another.

5.2.11 Response of *exoX* and *exoY* Promoters to Variations in pH

The β-galactosidase activities associated with four fusion clones were examined under acidic, neutral and basic conditions in the wild-type strain ANU240 background. The plasmid constructs were: pJG54 (*exoY'–lacZ*$^+$), pJG66 (ORF1'–*lacZ*$^+$), pJG60 (*exoX'–lacZ*$^+$), pARI7 (putative 2895'–*lacZ*$^+$). The strains carrying the constructs were cultured to early log phase in liquid MX media with 0.5 g.l^{-1} yeast extract, 0.5 g.l^{-1} Na-glutamate and 3 g.l^{-1} mannitol as carbon sources. The cultures were then dispensed into baffled flasks and the pH conditions adjusted (with KOH or HCl). The concentration of β-galactosidase was assayed after induction periods between 3 hr and 8 hr and either with and without neutralizing the pH. There was no difference in the activities from the promoters after a pH induction and neutralizing the pH prior to the β-galactosidase assay had no effect. Within the pH range examined, there were no dramatic regulatory effects on the promoters (Figs. 5.17A,B,E and F). The varied increases in cell density after a further 3 hr induction period demonstrates the effect of the various pH levels on general cell growth (Figs. 5.17C,D,G and H). The most acidic conditions were sufficiently severe to arrest cell division and the most basic conditions examined were able to decrease the amount of cell division by approximately 50%. Although the range of basic pH conditions was not as extreme as the acidic range, it is generally believed that the bacteroid compartment surrounded by the peribacteroid membrane, is likely to be acidic

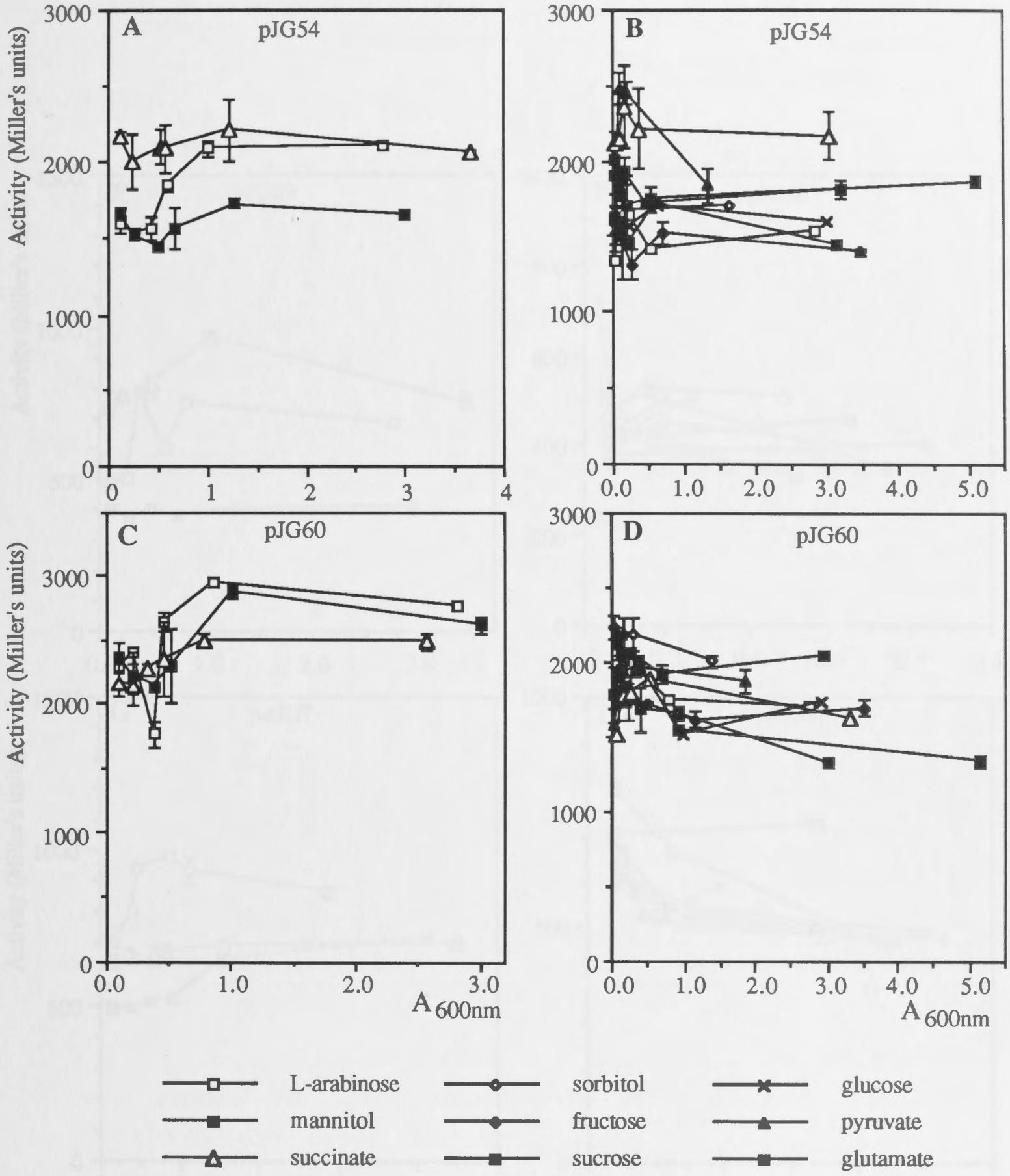


Fig. 5.16 Expression of *exo* promoters in strain ANU240, grown with different carbon sources. The β-galactosidase activity is represented in the units as defined by Miller (1972). **(A)** and **(B)** are repeat experiments with pJG54 (*exoY'-lacZ*$^+$). **(C)** and **(D)** are repeat experiments with pJG60 (*exoX'-lacZ*$^+$). **(E)** pJG66 (ORF1'-*lacZ*$^+$). **(F)** pJG58 (*exoY'-lacZ*$^+$). **(G)** and **(H)** are repeat experiments with pARI7 (putative 2895'-*lacZ*$^+$). Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

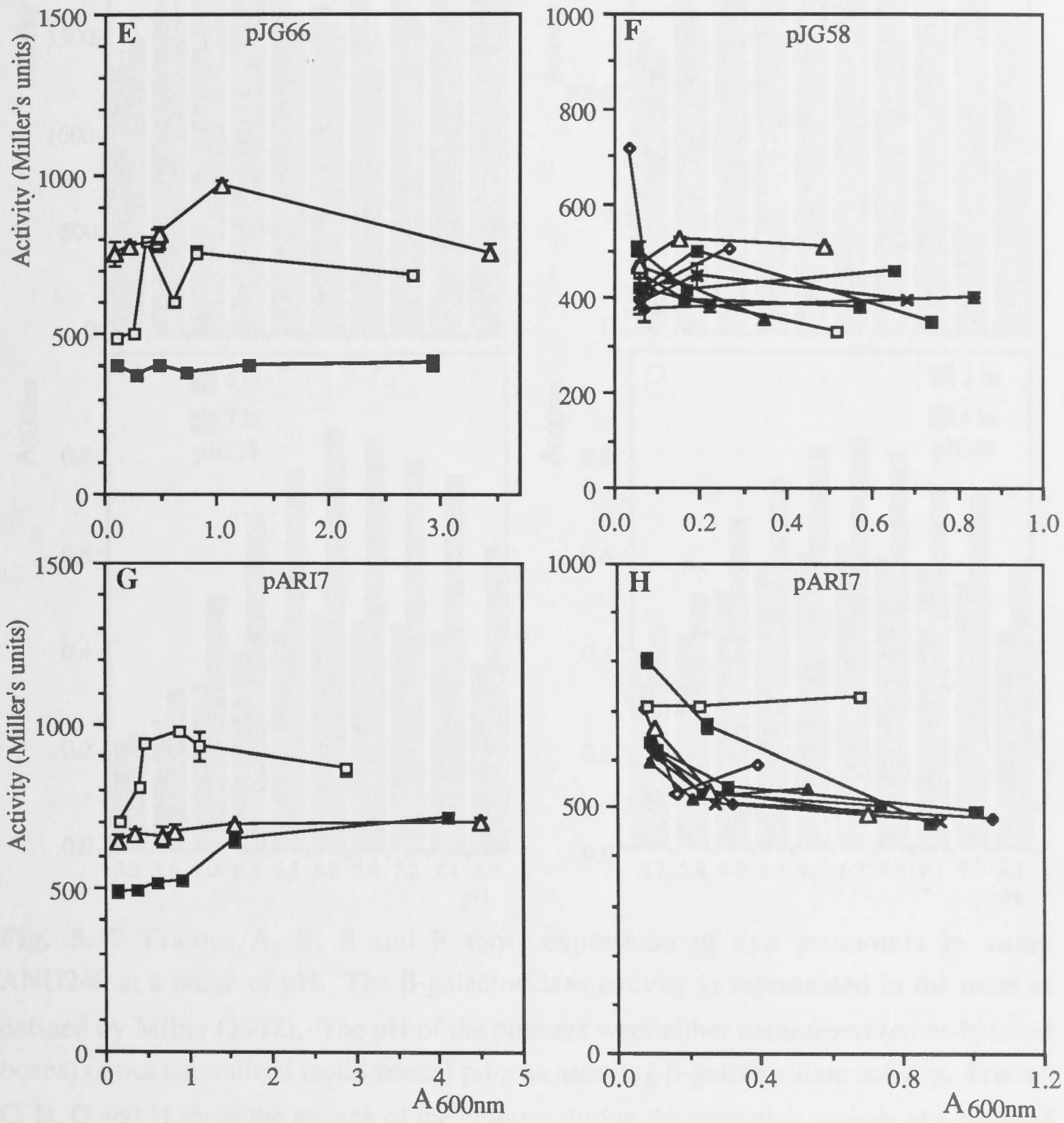


Fig. 5.16 continued

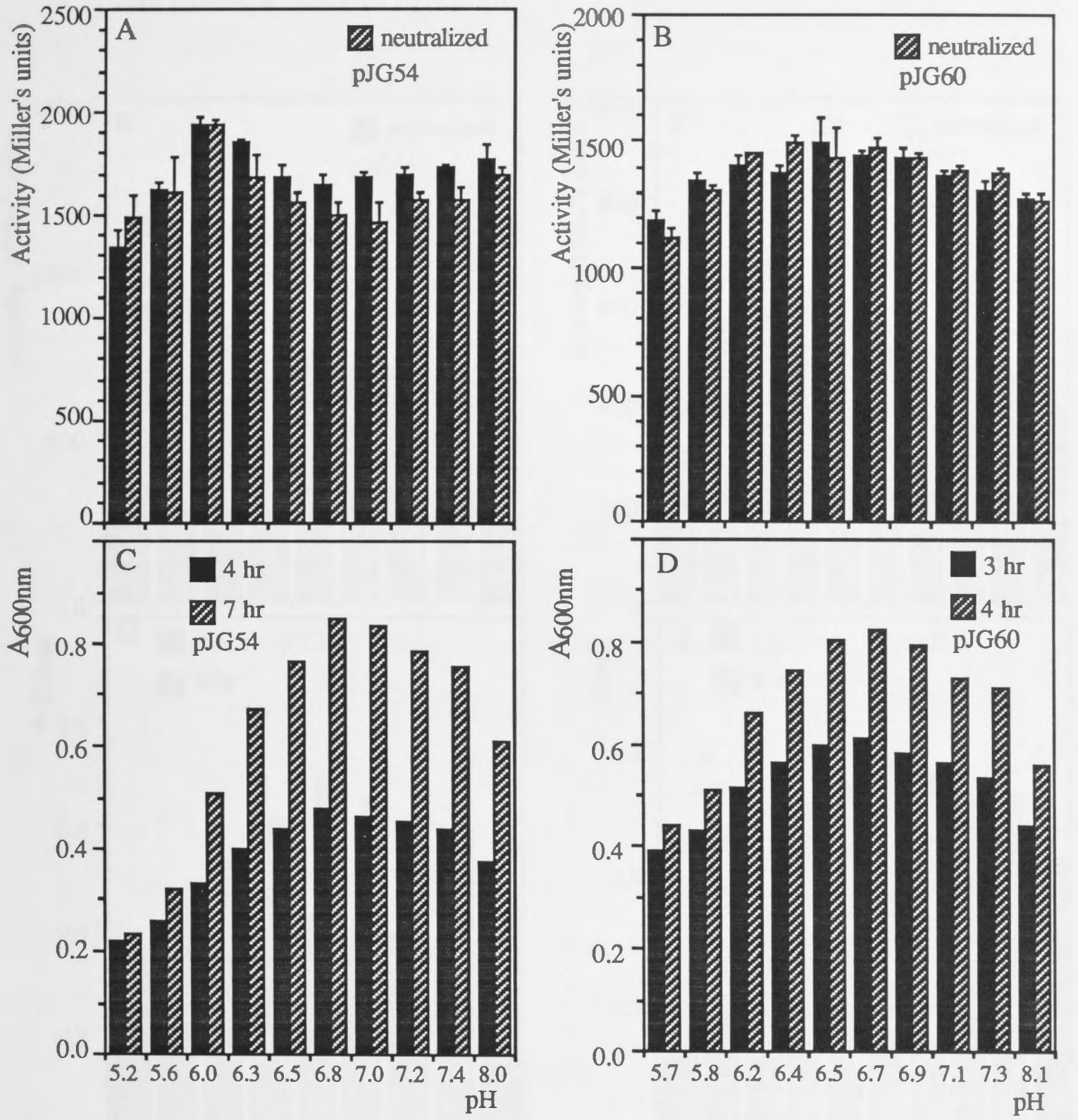


Fig. 5.17 Frames A, B, E and F show expression of *exo* promoters in strain ANU240 at a range of pH. The β-galactosidase activity is represented in the units as defined by Miller (1972). The pH of the cultures were either neutralized (cross-hatched boxes) or not neutralized (solid boxes) prior to assaying β-galactosidase activity. Frames C, D, G and H show the growth of the cultures during the induction periods at a range of pH. Induction periods varied and are shown in separate legends within each frame. Frames **(A)** and **(C)** are pJG54 (*exoY'-lacZ*$^+$). Frames **(B)** and **(D)** are pJG60 (*exoX'-lacZ*$^+$). Frames **(E)** and **(G)** are pJG66 (ORF1'-*lacZ*$^+$). Frames **(F)** and **(H)** are pARI7 (putative 2895'-*lacZ*$^+$). Each plot represents the mean of three observations. Error bars represent one standard deviation.

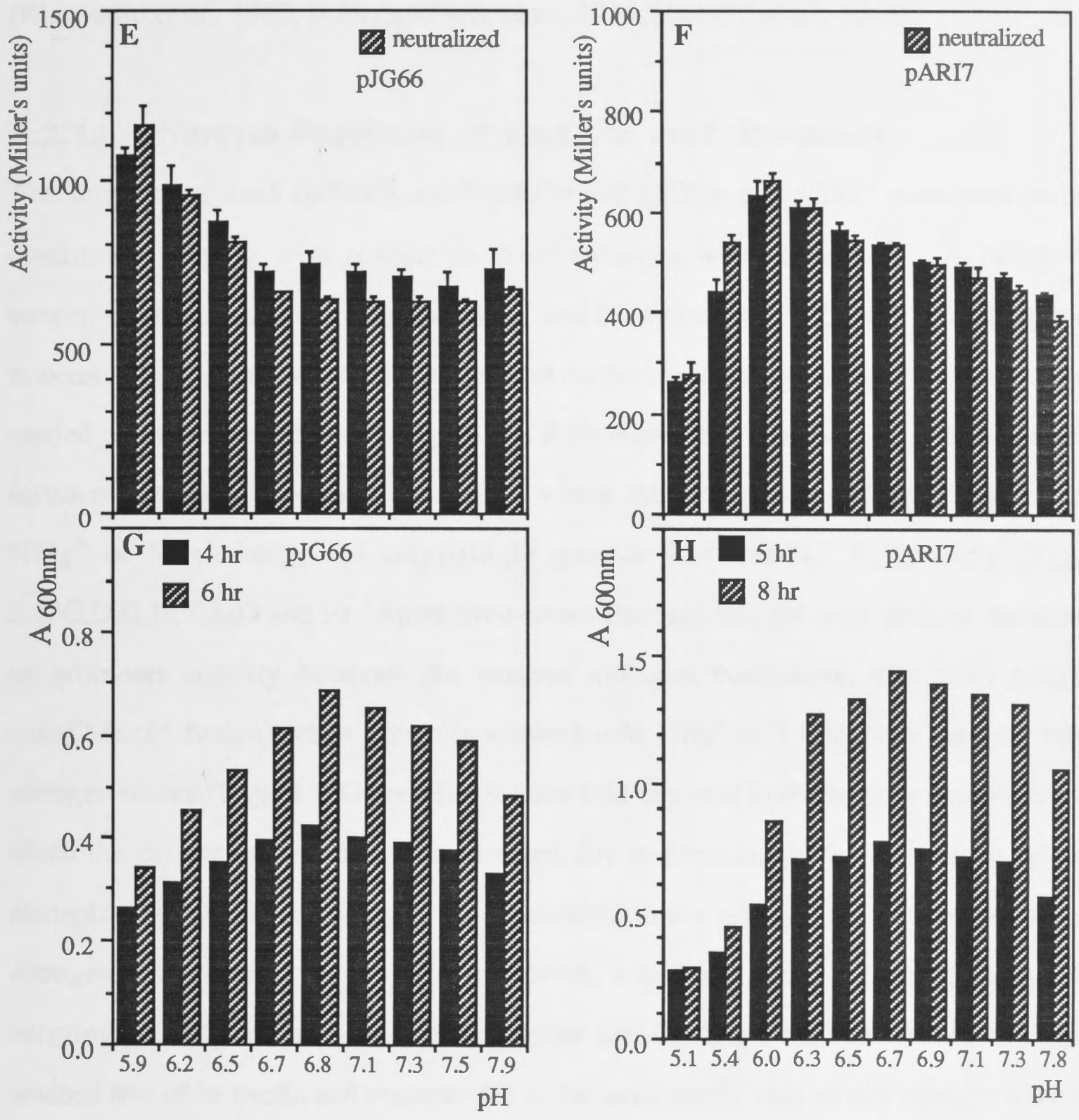


Fig. 5.17 continued

through the activities of proton-ATPases and transport systems for dicarboxylic acids (Blumwald *et al.,* 1985; Boller and Wiemken, 1986; Udvardi *et al.,* 1988a).

5.2.12 Nitrogen Regulation of *exoX* and *exoY* Promoters

The activities of *exoX* (pJG60), *exoY* (pJG54 and pJG66) and pARI7 promoters under conditions varying with respect to fixed nitrogen were examined. A range of concentrations of sodium glutamate, NH_4^+ and NO_3^- ions were all tested separately and in combination, to see what effects they had on the expression of these promoters when carried by the wild-type strain (Fig. 5.18). *Rhizobium* cells were able to utilize all three forms of nitrogen. However, in cultures where the nitrogen was limiting (eg. 1 mM NH_4^+ or NO_3^- ions), not surprisingly growth was arrested fairly early (Figs. 5.18C,D,G,H,K,L,O and P). Apart from minor fluctuations, the only striking variation in promoter activity between the various nitrogen conditions, was with pJG60 (*exoX'-lacZ*$^+$ fusion) when grown in either 1 mM NH_4^+ or 1 mM NO_3^- as their sole nitrogen source (Figs. 5.18G and H). A three fold increase in the activity was observed when the culture growth had been arrested due to presumably the exhaustion of the nitrogen supply. To investigate the possibility that a nearly total absence of fixed nitrogen is a stimulus for the *exoX* promoter, a mid-log phase culture (A_{600} = 1.4) carrying pJG60 that had been growing under non-limiting NH_4^+ conditions and then washed free of its media and resuspended in the same media void of any nitrogen source. These cultures were then grown in a range of low concentrations of NH_4^+ ions to monitor any increases in activity of the *exoX* promoter as the nitrogen supplies are sequentially exhausted (Fig. 5.19). No such increases in activity were observed from this more comprehensive investigation of low nitrogen levels. Slight increases in promoter activity were observed when nitrogen levels were 100mM (Fig. 5.18), but these concentrations are in excess of biologically attainable levels and thus was not considered a likely environmental stimulus.

Fig. 5.18 Expression of *exo* promoters in strain ANU240, grown in a variety of different fixed nitrogen sources at a range of concentrations. The β-galactosidase activity is represented in the units as defined by Miller (1972).

(A) pJG54 (*exoY'-lacZ*$^+$) with 10 mM Na-glutamate and a range of [NH_4^+].
(B) pJG54 with 10 mM Na-glutamate and a range of [NO_3^-].
(C) pJG54 with a range of [NH_4^+].
(D) pJG54 with a range of [NO_3^-].

(E) pJG60 (*exoX'-lacZ*$^+$) with 10 mM Na-glutamate and a range of [NH_4^+].
(F) pJG60 with 10 mM Na-glutamate and a range of [NO_3^-].
(G) pJG60 with a range of [NH_4^+].
(H) pJG60 with a range of [NO_3^-].

(I) pJG66 (ORF1'-*lacZ*$^+$) with 10 mM Na-glutamate and a range of [NH_4^+].
(J) pJG66 with 10 mM Na-glutamate and a range of [NO_3^-].
(K) pJG66 with a range of [NH_4^+].
(L) pJG66 with a range of [NO_3^-].

(M) pARI7 (putative 2895'-*lacZ*$^+$) with 10 mM Na-glutamate and a range of [NH_4^+].
(N) pARI7 with 10 mM Na-glutamate and a range of [NO_3^-].
(O) pARI7 with a range of [NH_4^+].
(P) pARI7 with a range of [NO_3^-].

Each plotted point represents the mean of three observations. Error bars represent one standard deviation and where not visible are smaller than the symbol.

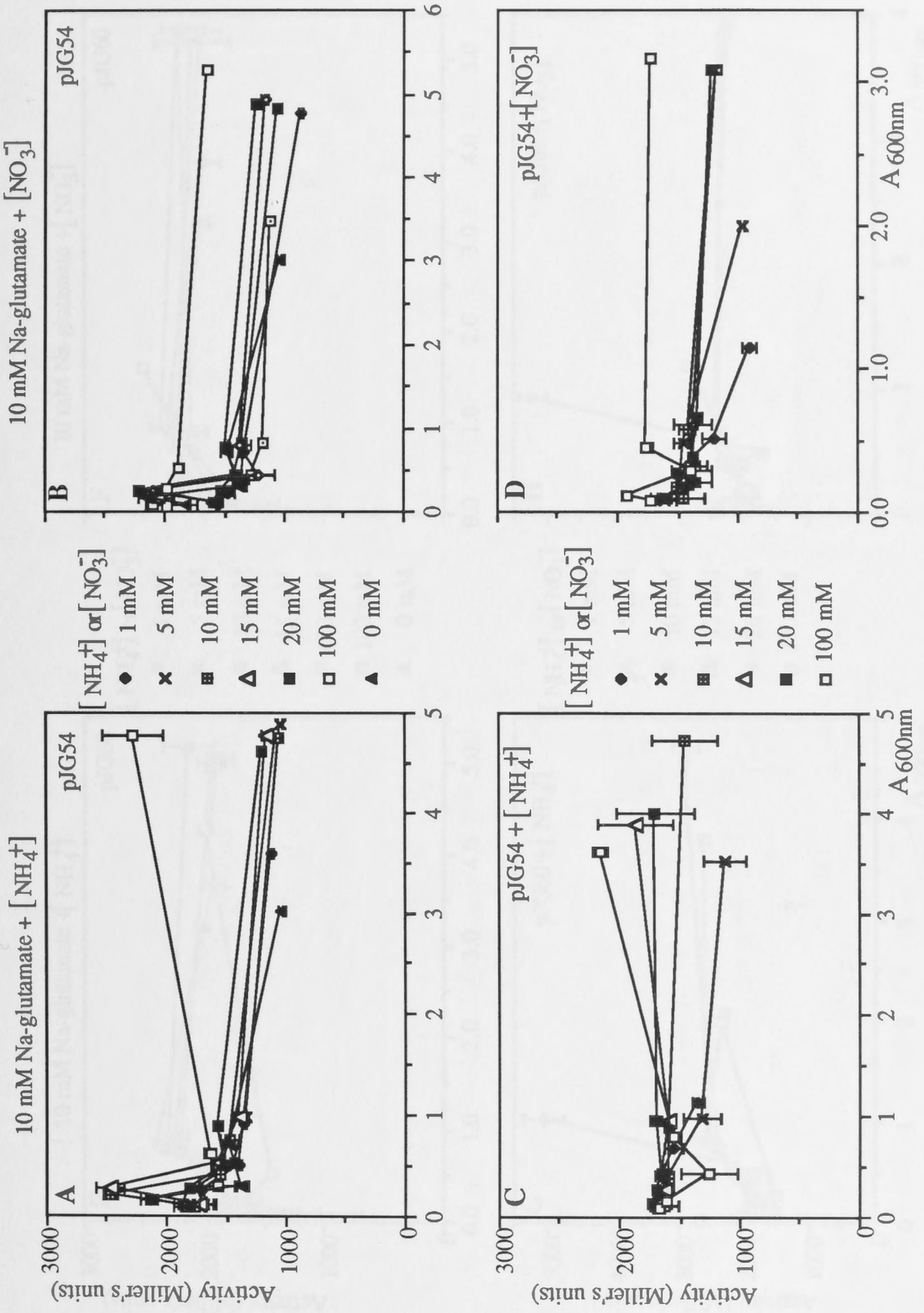


Fig. 5.18 continued

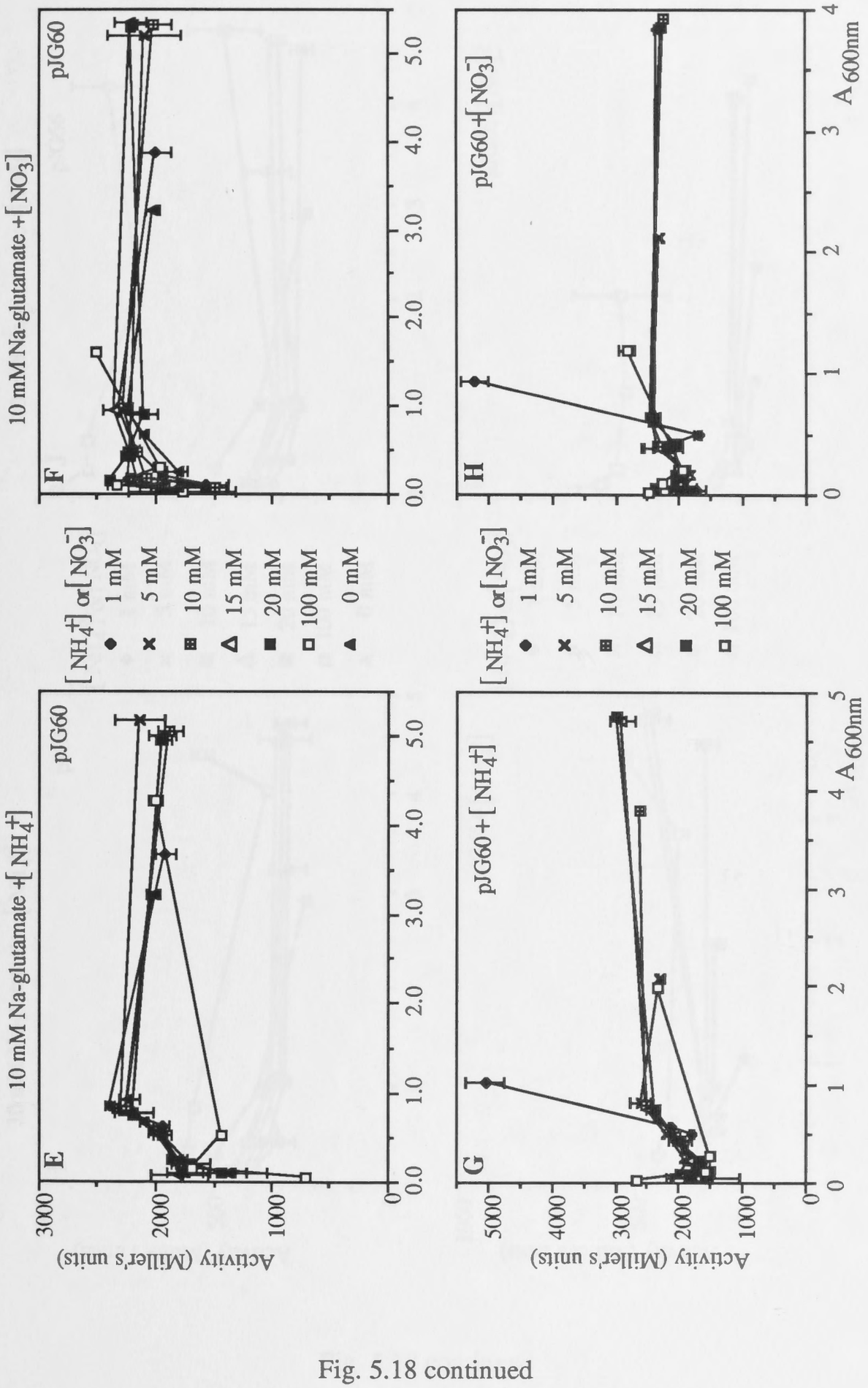


Fig. 5.18 continued

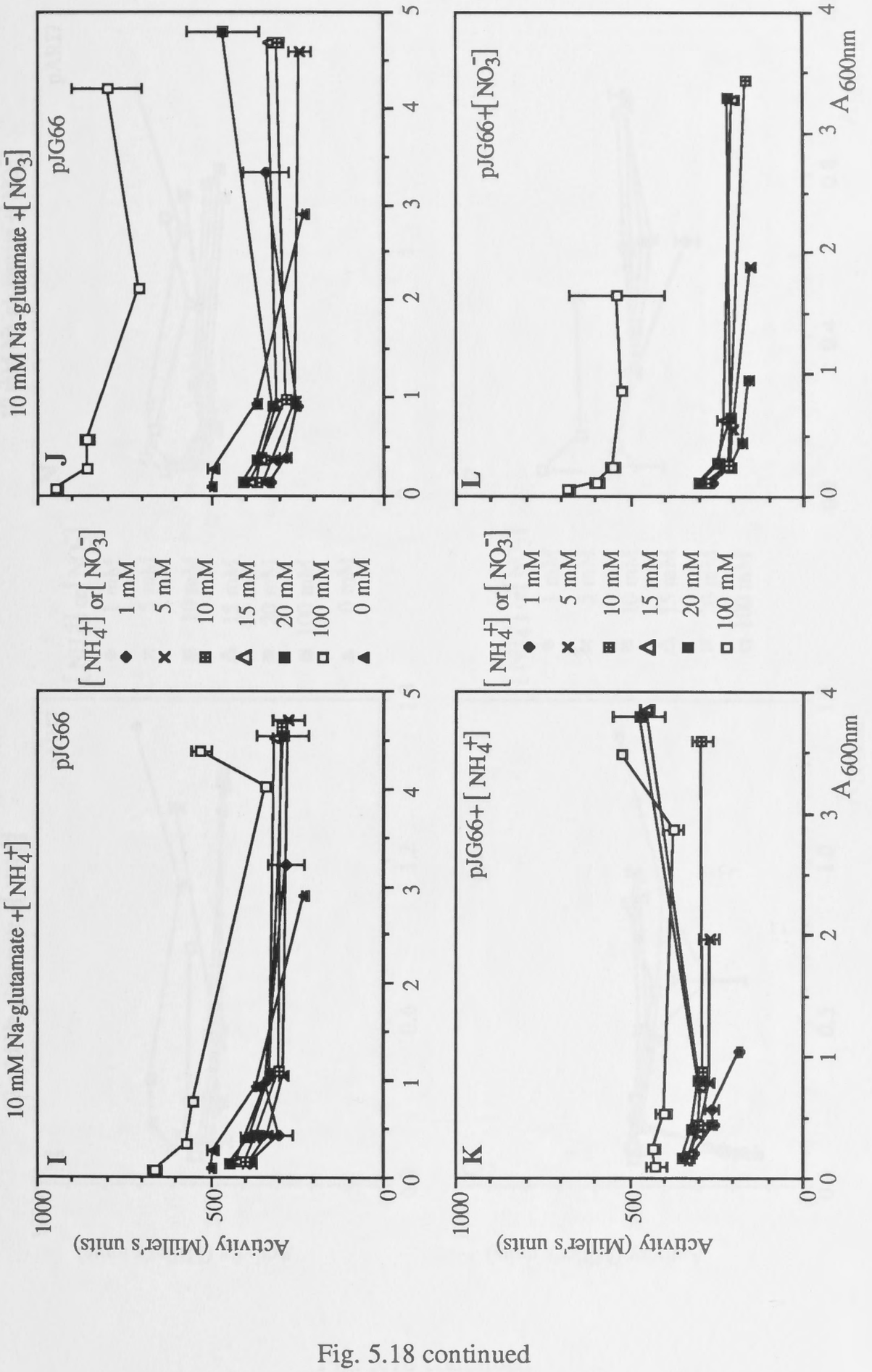


Fig. 5.18 continued

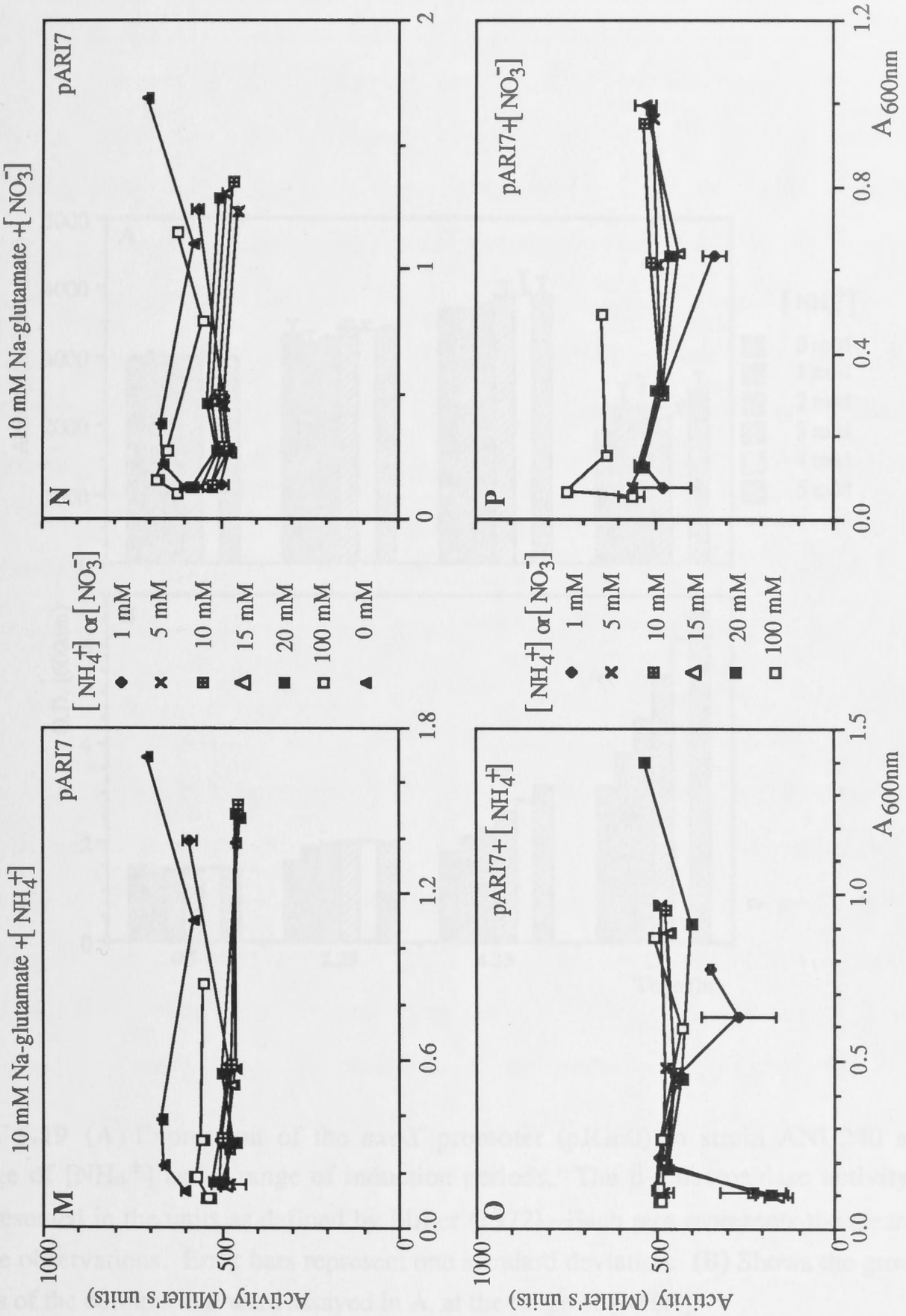


Fig. 5.18 continued

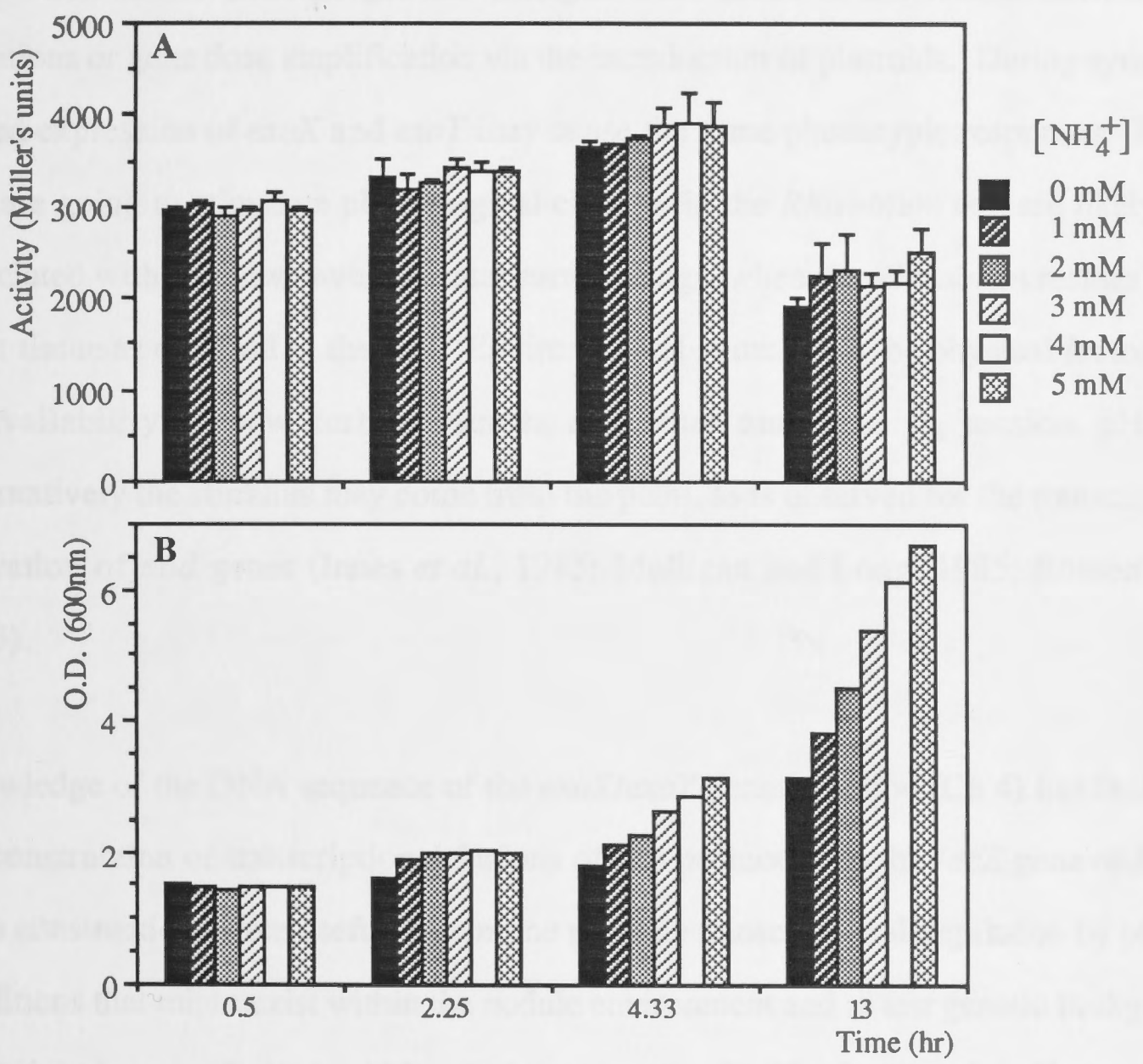


Fig. 5.19 **(A)** Expression of the *exoX* promoter (pJG60) in strain ANU240 at a range of $[NH_4^+]$ and a range of induction periods. The β-galactosidase activity is represented in the units as defined by Miller (1972). Each plot represents the mean of three observations. Error bars represent one standard deviation. **(B)** Shows the growth rates of the cultures that were assayed in A, at the range of $[NH_4^+]$.

5.3 DISCUSSION

From chapter 4 it is evident that the two genes, *exoX* and *exoY*, function as a binary regulatory system, that modulates the levels of EPS synthesis. Their effects were only seen when rather extreme genetic changes were made to the *Rhizobium*, such as mutations or gene dose amplification via the introduction of plasmids. During symbiosis, varied expression of *exoX* and *exoY* may cause the same phenotypic responses. Factors that are going to stimulate physiological changes in the *Rhizobium* cell are likely to be associated with the new environmental surroundings, when the *Rhizobium* resides within plant tissue as opposed to the soil. Environmental stimuli may be physical factors such as availability of new carbon sources and other nutrients, O_2 tension, pH, *etc*. Alternatively the stimulus may come from the plant, as is observed for the transcriptional activation of *nod* genes (Innes *et al.*, 1985; Mulligan and Long, 1985; Rossen *et al.*, 1985).

Knowledge of the DNA sequence of the *exoX/exoY* genetic region (Ch 4) has facilitated the construction of transcriptional fusions of *exo* promoters to the *lacZ* gene of *E. coli*. Such constructions were useful to examine possible transcriptional regulation by physical conditions that might exist within the nodule environment and to test genetic backgrounds that might have mutations within regulatory genes. In this chapter, the effects of fixed nitrogen forms and availability, sources of carbon supply, pH, O_2 availability, and a variety of genetic backgrounds were all examined for their possible transcriptional regulatory effects on the promoters of *exoX, exoY* and another promoter in the vicinity of the 2895::Tn*5* *exo* mutation. All regulation studies were conducted using *R*. sp. NGR234 strains grown in liquid cultures. Although it is not possible to simulate a nodule environment, any single factors that affect transcription of the test promoters will still have their influence when artificially applied to a culture.

From all of the genetic backgrounds examined, the only significant regulatory effect was with the promoter of the *exoY*-ORF1 operon in the background of Tn*5* mutant strain ANU2895. Several *exoY* and ORF1 transcriptional fusions were examined and they all showed increases in activity between 2.5 and 5 fold when tested within the strain ANU2895 background compared to the wild-type and other mutant backgrounds. The colony morphology of strain ANU2895 is very mucoid (Exo^{++}) and estimates of hexose content by anthrone-H_2SO_4 assays indicate that a strain ANU2895 culture produces EPS at a level 2.8 times greater than that produced by the wild-type strain ANU280. The mutation in strain ANU2895 has resulted in the deregulation of EPS biosynthesis and therefore, the mutated locus is probably a regulatory gene that normally functions as a repressor. The data supports the model that the 2895 gene encodes a *trans*-acting repressor that in the wild-type would negatively regulate transcription of *exoY*. When the repressor is absent, as in the case of strain ANU2895, the over expression of *exoY* would lead to increased EPS production. Furthermore, when multiple copies of *exoY* in the absence of *exoX* are transferred to wild-type strains, the colony morphology of the transconjugants is similar to those of strain ANU2895 (Exo^{++}). This negative regulation of the *exoY* promoter may be required in order to maintain the critical balance between *exoY* and *exoX* gene products, which is necessary for properly regulated EPS production. A similar regulatory link was shown to exist in *R. meliloti* by Doherty *et al.* (1988). Expression of two genes, *exoF* and *exoP*, were shown to be transcriptionally deregulated in mutant backgrounds of *exoR*::Tn*5* and *exoS*::Tn*5* (Doherty *et al.*, 1988). Mutations in *exoR* and *exoS* result in the over-production of EPS (Doherty *et al.*, 1988) and generate colonies with a similar morphology to those of strain ANU2895. Furthermore, *exoF* is believed to be the *R. meliloti* homolog to *exoY* (see chapter 6).

The possibility that the inhibitory effects of *exoX* are counteracted by *exoY*, via a system where *exoY* represses the transcription of *exoX*, was not found to be the case. The activity of the *exoX* promoter was equal in both strain ANU280 (wild-type) and strain

ANU2811 (*exoY*::Tn*5*) backgrounds. In fact, the activity of *exoX* was not found to be different in any of the *exo* mutant backgrounds examined. The possibility that multiple copies of *exoX* inhibits EPS, via a system where it represses transcription of *exoY*, is also not the case. Transcriptional fusion constructs of the *exoY* promoter to *lacZ*, where the entire *exoX* gene was also present on the plasmid, still showed very high activity from the *exoY* promoter despite the fact that EPS production by the transconjugants was inhibited. These experiments support a model that the *exoX*/*exoY* regulation of EPS occurs at a post-translational level (discussed further in chapters 6 and 7).

These experiments involved comparing promoter activity in backgrounds that had differing Exo phenotypes. Thus it was necessary to investigate the effects that EPS had on spectrophotometric determinations of optical densities for cell cultures, because part of the Miller (1972) equation for determining β-galactosidase activity involves normalizing the total activity of a sample by its cell density. It was found that less light was absorbed by cells that had more encapsulating EPS. This experiment was repeated several times, since the reverse was anticipated. Consistently, more protein and CFUs were recovered from strain ANU2895 (Exo^{++}) cultures as from strain ANU280 (Exo^{+}, wild-type) cultures that were made to have the same A_{600} values and similarly between strain ANU280 and strain ANU2811 (Exo^{-}, *exoY*::Tn*5*). It would appear that the tendency for light to be refracted or reflected rather than absorbed by cells, is directly proportional to the amount of encapsulating EPS. Thus, in comparisons involving promoter activities between strains of different Exo phenotypes, it was necessary to introduce multiplication factors into the Miller activity equation to compensate for the effects of EPS.

Whenever possible, the activity from promoter constructs was assayed at various stages throughout a growing culture, because interpretations from a curve were more reliable than from a single value. It was necessary to include antibiotic selection for the promoter fusion plasmids in order to maintain the copy number at a high level. While the plasmid

was rarely entirely lost in the absence of antibiotic selection, the β-galactosidase activity would none the less decrease logarithmically with cell growth, indicating that the copy number of the plasmid per cell was definitely decreasing with cell division.

The promoters tested showed no regulation by *nodD* or any other genetic loci present on the symbiotic plasmid (pSym). This is not surprising, since the EPS produced by strain ANU265 (pSym cured derivative of *R*. sp. NGR234) showed no differences in its EPS structure or in the amounts produced, compared with the wild-type strain (Djordjevic *et al*., 1987c).

The amount of EPS production per cell is constant through out all stages of a growing batch culture. Estimations of the hexose content of liquid *R*. sp. NGR234 strain ANU240 cultures, indicated that the amount of EPS present was directly proportional to the amount of protein (*ie*. cell density). Thus, EPS production is dependent upon metabolic rates of the cell and the availability of nutrients, in the same way as protein synthesis and cell division. This observation was important when studying the effects of low O_2 availability on EPS production. The production of EPS by strain ANU240 was not inhibited under anaerobic conditions as has been reported for *B. japonicum* (Tully and Terry, 1985). Liquid cultures of strain ANU240 were grown under conditions of varying degrees of O_2 limitation. In all cases, the time taken for cultures to attain an adequate cell density for the anthrone-H_2SO_4 assay was inversely proportional to the concentration of O_2 in the gas phase. As the results indicated, the amount of EPS produced per cell was also inversely proportional to the concentration of O_2 availability. Thus, it is evident that EPS production still occurs even when protein synthesis and cell division are restricted due to limited O_2 availability. The fact that more EPS per cell was recovered from cultures where growth was restricted, was probably due to the fact that these cultures had longer generation times, but still continued to synthesize EPS at a similar rate per hour. It should be noted that EPS production per cell, in this experiment,

was not dependent on the metabolic rates of the cell. This is because under O_2 limiting conditions all other nutrients and substrates (most importantly the carbon source) are in abundant supply, in contrast, many of these are exhausted in the stationary phase of a batch culture. Bacterial production of EPS does not require the culture to be dividing and growing. EPS produced for industrial purposes are often synthesized in a single stage continuous culture where it is vital to have the carbon source in excess, but either the nitrogen or sulfur source limited in order to restrict protein synthesis. In addition, there were no differences in expression from the cloned *exo* promoters, when cultures carrying the fusion constructs were assayed after incubation periods in a range of O_2 limiting conditions.

During early stages of infection the *Rhizobium* cells would be exposed to a variety of new carbon sources found in the plant, such as sucrose, fructose, L-arabinose, other sugars and polyalcohols (Streeter, 1981). However, in the symbiotic state, the *Rhizobium* cells are exposed to a very limited range of carbon sources. All nutrients destined for the bacteroid cells must pass through the peribacteroid membrane, which is a selective permeability barrier for various forms of carbon, permitting only the passage of dicarboxylic acids (Price *et al.*, 1987). In addition, the dicarboxylic acid, succinate, was able to induce bacteroid-like traits on a free living culture of *R. trifolii* strain 0403 (Urban and Dazzo, 1982). Succinate induced these cells to stop dividing *in vitro* and to swell, and their morphology closely resembled that of bacteroids in nitrogen-fixing nodules on white clover. Since it is not known precisely when EPS production may be regulated, a range of hexoses, polyalcohols and dicarboxylic acids were tested for their possible transcriptional effects on the *exo* promoters. The expression of the *exo* promoters did not vary dramatically with any of the carbon sources tested. Similarly, the pH of the growth medium was not found to influence the expression of the *exo* promoters, despite its potential as a stimulus due to the inevitable difference in pH that would exist in the plant tissue as compared to the soil.

The availability of fixed-nitrogen was not a regulatory stimulus for the tested *exoY*-ORF1 or 2895 *exo* promoters, but the expression of *exoX* may be influenced by fixed nitrogen levels. The induction of the *exoX* promoter in response to essentially zero fixed nitrogen levels was investigated in to ways (section 5.2.12), which differed with respect to the number of cell divisions that occurred during the induction period. First, an ANU240(pJG60) culture was grown with a very low concentration of available fixed nitrogen (1 mM) from a small inoculum. Under these conditions, the activity of the *exoX* promoter increased 2.5 fold when the fixed nitrogen source was exhausted (after approximately 12 to 15 generations), as indicated by the concurrent arresting of cell division. However, the second experiment which showed no variation in *exoX* expression, allowed the ANU240(pJG60) culture to grow initially with a non-limiting supply of fixed nitrogen and then the growth medium was abruptly replaced with one that had limited levels of fixed nitrogen. In this experiment, the number of cell divisions that occurred before growth was arrested, was far fewer (*eg*. 2 cell divisions for 0 mM NH_4^+, Fig. 5.19B) compared to the first experiment. Therefore, the increased expression of *exoX* may not result from a simple induction, but may require several cell divisions in order to condition the *Rhizobium* cells; such as would be expected if the cellular concentration of a putative *exoX* repressor was required to be depleted below a threshold level.

In *R. meliloti* the concentration of NH_4^+ ions has been implicated in the regulation of EPS, mediated through the gene *exoR* (Doherty *et al*. 1988). Where as the amount of EPS produced by the wild-type *R. meliloti* is increased 23 fold under conditions where the concentration of NH_4Cl is limiting, *exoR*::Tn*5* mutants did not show any significant increase in EPS production under the same NH_4Cl limiting conditions compared to non-limiting conditions. Regardless of the NH_4Cl concentration, *exoR*::Tn*5* mutants produced EPS at a level 224 fold higher than the wild-type. It is however possible that in

these experiments (Doherty *et al.,* 1988), the amount of extra EPS production per cell under NH_4Cl limited growth was due to the arresting of protein synthesis and cell division without affecting the EPS synthesis to the same degree. Unlike protein synthesis, only adequate carbon and not nitrogen is required for EPS biosynthesis (Duguid and Wilkinson, 1953). In addition, the lack of any further increase in EPS production by *exoR*::Tn*5* mutants under NH_4Cl limited growth may be because the maximal level has already been attained, due to the depletion of substrate or degradation of the overworked enzymes.

The transcriptional fusions of *exo* promoters to *lacZ* was a good system for screening large numbers of *exo* mutant strains in order to identify *trans*-acting genetic elements affecting expression from these cloned promoters. Through this approach, the locus mutated in strain ANU2895 was identified as a *trans*-acting repressor of *exoY*. It was assumed that the same fusion constructs could be used in a wild-type background in order to investigate the regulatory effects of a range of physical environmental growth conditions. The transformation from free-living *Rhizobium* to bacteroid *Rhizobium* is marked by many morphological, physiological and biochemical changes, and it was appreciated that this differentiation would require a range of precise environmental stimuli. However, since these fusion constructs are monitoring only a single putative symbioticly regulated promoter, it was conceivable that any one promoter may be influenced by only a single environmental stimulus. However, such a regulatory link with growth conditions was not found in this study.

Legumes are known to release large amounts of flavanoid compounds, that stimulate expression of the *Rhizobium* nodulation genes via their interaction through *nodD* (Redmond *et al.*, 1986; Peters and Long, 1988). These small molecular weight organic molecules are exuded from the plant into the surrounds, where they act as signal molecules for the *Rhizobium* cells. Similarly, *Rhizobium* exopolysaccharide

biosynthesis may be regulated by signals originating from the plant. However, since EPS is required for early interactions of rhizobia and host and is mandatory for normal development of an indeterminate nodule, any repression of EPS synthesis would occur at a late stage in nodule development. Regulatory molecules of plant origin affecting *Rhizobium* EPS production, are not likely to be released beyond the plant tissue as this would interfere with early interactions. The regulatory molecules may be of relatively large size, perhaps proteins, and act on *Rhizobium* cells in infection threads or within peribacteroid units. I believe that before one sets about attempting to isolate stable, bioactive protein extracts from plants, it would be better to examine the test promoter in an *in situ* assay system utilizing the reporter gene GUS (Jefferson *et al.*, 1987). Although the *in situ* system would not discern between regulation due to physical conditions or a plant signal, it would at least establish whether these promoters are regulated in the plant environment.

Acknowledgement

The plasmid, pARI7, and information used to prepare figure 5.2 was provided by Tony Arioli; and is appreciated.

CHAPTER SIX

Relatedness of *exo* genes Among *Rhizobium* Species

6.1 INTRODUCTION

As all *Rhizobium* species and many other soil bacteria synthesize exopolysaccharides, the prevalence of DNA sequences with homology to the cloned *R*. sp. NGR234 *exo* DNA was investigated in a variety of bacterial species. Sequence homology and functional interchangeability of *exo* genes was found between *R*. sp. NGR234 and *R. meliloti*. Elucidations of the genes involved in EPS synthesis is fairly advanced for both of these two *Rhizobium* species, and thus further knowledge about EPS biosynthesis could be gained by constructing genetic hybrids. In addition, the responses of host plants to hybrid constructs that synthesize heterologous EPS structures will yield information pertaining to the chemical signal properties of EPS and the specificity shown by host plants to these molecules.

The structures of acidic EPS from *R. meliloti* and *R*. sp. NGR234 have a region of similarity, while the remainder of the molecules are unique. *R*. sp. NGR234 produces an EPS that has a nonasaccharide repeat unit, which contains five glucoses, two galactoses and two glucuronic acids, all in various α and β linkages, and one pyruvate and one acetate group (Djordjevic *et al.*, 1986). *R. meliloti* EPS (succinoglycan) on the other hand, has an octasaccharide repeat unit, that contains seven glucoses and one galactose, all in various β linkages, as well as one pyruvate, one acetate and one succinate group (Aman *et al.*, 1981). Both oligosaccharide repeat units have a common region of one galactose and four glucoses, all with the same β linkages (the structures of the two oligosaccharide repeat units are shown in Fig. 1.1). Synthesis of succinoglycan begins with the transfer of a galactose residue to the lipid carrier followed then by the remaining seven glucosyl units in sequential transferase steps (Tolmasky *et al.*, 1980 and 1982). It is possible that synthesis of the *R*. sp. NGR234 EPS oligosaccharide repeat

unit also begins with the galactose sugar and if so, perhaps the first five transferase steps in the assembly of both repeat units are processed by the products of homologous genes. The remaining sugar residues are unique between the two repeat unit structures and they both have non-carbohydrate substitutions at different sites. Thus, while some genes may be functionally interchangeable, the two *Rhizobium* species will certainly have unique *exo* genes as well.

6.2 RESULTS

6.2.1 Inhibition of Exopolysaccharide Synthesis by *exoX* and *nodD2*

In section 4.2.4, it was reported that the plasmid pJG22::Tn*5* (carries a wild-type *exoX* and a mutant *exoY*::Tn*5*) will confer an Exo$^-$ phenotype on the wild-type ANU280 strain and in section 4.2.7, the same result was observed for pJG53 (carries *exoX* and a truncated *exoY*). It was shown that elevated copies of *exoX* relative to *exoY* resulted in the Exo$^-$ phenotype. To investigate whether *exoX* has a similar activity in a range of other soil bacteria, pJG53 and pJG22::Tn*5* were transferred into *R. meliloti* strain Rm1021, *R. fredii* strain USDA191, *R. l.* bv. *trifolii* strain ANU843 (wild-type), *R. l.* bv. *trifolii* strain ANU845 (pSym$^-$), *A. tumefaciens* strain C58 (wild-type) and *A. tumefaciens* strain GMI9023 (pTi$^-$, pAt$^-$). These bacterial strains have Exo$^+$ colony morphologies on solid BMM media. A dry Exo$^-$ colony morphology for transconjugants carrying pJG53 or pJG22::Tn*5* was interpreted as *exoX* activity in the foreign background. Plasmids pJG53 and pJG22::Tn*5* were able to inhibit EPS synthesis in *R. meliloti* and *R. fredii* transconjugant strains, but not *R. l.* bv *trifolii* or *A. tumefaciens* transconjugant strains (Table 6.1).

Interestingly, another plasmid pRK14-17, was able to inhibit EPS synthesis by strain ANU280, generating an Exo$^-$ colony morphology indistinguishable from those caused by an *exoX* clone (Table 6.1). Plasmid pRK14-17 carries the wild-type *nodD2* gene from *R. fredii* and it was shown to inhibit EPS synthesis by *R. fredii* transconjugants that

harboured this plasmid (Appelbaum *et al.*, 1988) . A derivative of pRK14-17, which has a Tn*5* located within the *nodD2* gene (pRK14-17K1) did not have any affect on the EPS synthesis by *R. fredii* transconjugants (Appelbaum *et al.*, 1988) or by strain ANU280 transconjugants (Table 6.1). The *nodD2* gene has no homology with *exoX*, although it possibly acts upon the same biosynthetic pathway as *exoX*, but at a different point.

Table 6.1 Inhibition of EPS synthesis by *exoX* and *nodD2*

Recipient strain	Transconjugant phenotype due to plasmid					
	pJG53 (*exoX*)	pJG22::Tn*5* (*exoX*)	pJG22 (*exoX,Y*)	pRK14-17 (*nodD2*)	pRK14-17K1 (*nodD2*::Tn*5*)	pMP220 (vector)
ANU280	Exo$^-$	Exo$^-$	Exo$^+$	Exo$^-$	Exo$^+$	Exo$^+$
Rm1021	Exo$^-$	Exo$^-$	Exo$^+$	-	-	Exo$^+$
USDA191	Exo$^-$	Exo$^-$	Exo$^+$	-	-	Exo$^+$
ANU843	Exo$^+$	Exo$^+$	Exo$^+$	-	-	Exo$^+$
ANU845	Exo$^+$	Exo$^+$	Exo$^+$	-	-	Exo$^+$
C58	Exo$^+$	Exo$^+$	Exo$^+$	-	-	Exo$^+$
GMI9023	Exo$^+$	Exo$^+$	Exo$^+$	-	-	Exo$^+$

" - " indicates that the experiment was not done.

6.2.2 Evidence that the *exoY* Promoter has a *nod*-box

The possibility that regulation or coordinated expression of *exo* genes occurs via a system analogous to the *nodD1* regulation of *nodABC*, was investigated using a *nod*-box specific, oligodeoxynucleotide probe in combination with analysis of the DNA sequence from the *exoX-exoY* region. Cloned *exo* DNA was probed using a 20-mer oligodeoxynucleotide specific for the *nod*-box of the *R.* sp. MPIK3030 *nodD1* promoter (Horvath *et al.*, 1987). Although strain MPIK3030 is not identical to strain NGR234, they appear to be derivatives of the same same parental strain (Bachem *et al.*, 1986) and in addition this same *nod*-box oligodeoxynucleotide probe was shown to hybridize to the *nodD1* promoter in *R.* sp. NGR234 (Bassam *et al.*, 1988). However, no hybridization of this oligodeoxynucleotide probe to any sequences within the 19 kb *exo* DNA region

was detected, although control experiments showing strong hybridization to the strain NGR234 *nodD1* sequence (data not presented) indicated that the hybridization conditions were satisfactory.

Analysis of the sequenced promoter regions of *exoX* and *exoY* operons, revealed the existence of two *nod*-box like sequences, at positions #849 and #940 of the nucleotide sequence (see chapter 4, Fig. 4.10). These *nod*-box-like sequences were located 100 bp and 190 bp upstream of the proposed *exoY* transcription initiation site. Figure 6.1 shows the nucleotide sequence of the two putative *nod*-boxes along side the *nodD1* *nod*-boxes from *R*. sp. MPIK3030 (Horvath *et al.*, 1987) and *R. meliloti* (Rostas *et al.*, 1986). The divergence between the MPIK3030 oligodeoxynucleotide sequence used as a probe and the two putative *nod*-box sequences, explained the lack of hybridization. The following is the number of conserved nucleotides, from a total length of 25 bp, between the four *nod*-boxes compared in figure 6.1.

	#940	MPIK3030	*R. meliloti*
#849	14	10	15
#940	-	12	14
MPIK3030	-	-	19

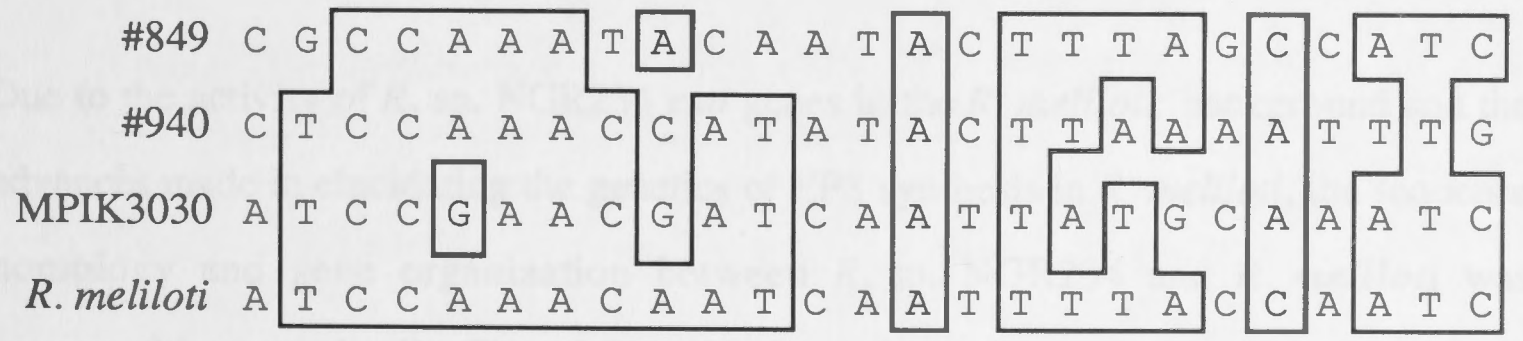


Fig. 6.1 Comparison of *nod*-box-like sequences. Two *nod*-box like sequences located at nucleotide positions #849 and #940 (Fig. 4.10), which is within the putative promoter for *exoY*, are compared to the *nod*-box sequences of *nodD1* promoters in *R*. sp. MPIK3030 (Horvath *et al.*, 1987) and *R*. meliloti (Rostas *et al.*, 1986). Nucleotides that are conserved between either one of the *exoY* putative *nod*-boxes and either one of the *nodD1* *nod*-boxes are enclosed within rectangles.

6.2.3 Hybridization Homology of *exo* DNA in a Range of Bacteria

Hybridization experiments were conducted using *R.* sp. NGR234 *exo* DNA to probe the genomes of a wide range of bacteria; including fast-growing *Rhizobium*, such as *R. l.* bv. *viciae* strain ANU300, *R. l.* bv. *trifolii* strain ANU843, *R. meliloti* strain Rm1021, *R. fredii* strain USDA191; slow-growing *Bradyrhizobium*, such as *B. japonicum* strain USDA110, *B. parasponia* strain ANU289, general cowpea *Bradyrhizobium* strain CB756; other bacteria such as *A. tumefaciens* strain C58, *Pseudomonas fluoresence* strain PFN018, *Klebsiella pneumoniae* strain KPN5, *Erwinia chrysanthenni* strain 507, *Lignobacter* sp. K17, *Azospirillum brasilense* strains SP7 and SP245, *Anabaena* sp. PCC7120, *Anabaena azollae microphilla* strain MIC2, *A. azolla pinnata* strain INDM and *E. coli* K12. Highly homologous sequences were found in the genomes of *R. meliloti* and *R. fredii* (Fig. 6.2), but not in the other *Rhizobium* species examined. With the exception of *A. tumefaciens*, hybridization to DNA sequences from any of the slow-growing rhizobia or other bacterial species was not detected, even when low stringency hybridization conditions were applied. DNA sequences within *A. tumefaciens* strains A136 (pTi^-) and GMI9023 (pTi^-, pAt^-), hybridized weakly to the *R.* sp. NGR234 *exo* probe DNA under low stringency conditions (55°C, 0.9 M NaCl) (Fig. 6.3). Thus, *A. tumefaciens* not only carries homologous sequences, but they are probably located on the chromosome.

Due to the activity of *R.* sp. NGR234 *exo* genes in the *R. meliloti* background and the advances made in elucidating the genetics of EPS synthesis in *R. meliloti*, the sequence homology and gene organization between *R.* sp. NGR234 and *R. meliloti* was compared in more detail. Cloned *R. meliloti exo* DNA from the five original cosmids, pD2, pD5, pD15, pD34 and pD56 (Leigh *et al.*, 1985), was probed with the cloned *R.* sp. NGR234 *exo* DNA (Fig. 6.4). The broad genetic organization between the two *Rhizobium* species, based upon the hybridization experiments, is summarized in figure 6.5. The 10 kb *Bam*HI insert of pJG11 hybridized strongly to the left-most *Eco*RI

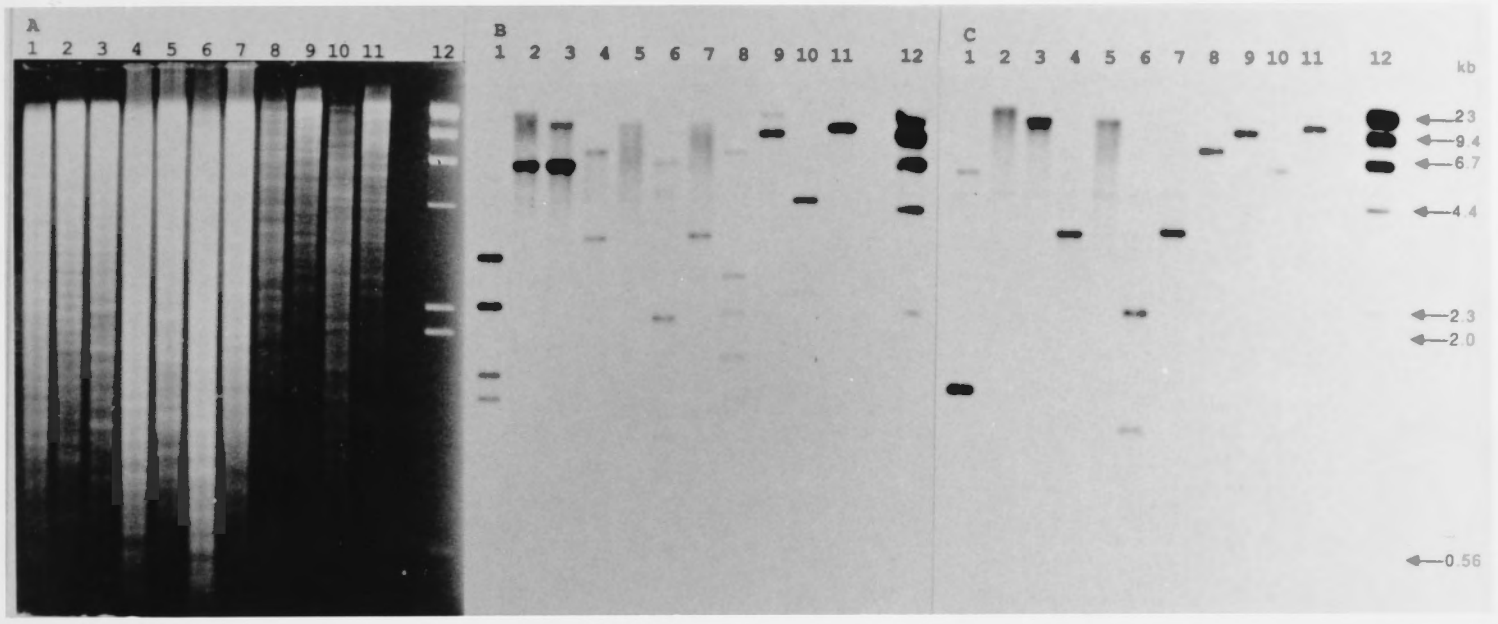


Fig. 6.2 Hybridization of *exo* DNA to *R. fredii* and *R. meliloti* genomic DNA. **(A)** Electrophoresis of restricted genomic DNA from three *Rhizobium* species, through a 0.8% agarose gel. **(B)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 10 kb *Bam*HI insert of pJG11. (C) Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 2.2 kb *Bam*HI-*Eco*RI terminal fragment of the pJG11 insert. Hybridization conditions were stringent (65°C, 0.9 M NaCl).

Lane 1 *R. fredii* strain USDA191 DNA restricted with *Eco*RI,
Lane 2 *R. fredii* strain USDA191 DNA restricted with *Hin*dIII,
Lane 3 *R. fredii* strain USDA191 DNA restricted with *Bam*HI,
Lane 4 *R. meliloti* strain Rm1021 DNA restricted with *Eco*RI,
Lane 5 *R. meliloti* strain Rm1021 DNA restricted with *Hin*dIII,
Lane 6 *R. meliloti* strain Rm1021 DNA restricted with *Pst*I,
Lane 7 *R. meliloti* strain Rm1021 DNA restricted with *Bam*HI,
Lane 8 *R. sp.* NGR234 strain ANU280 DNA restricted with *Eco*RI,
Lane 9 *R. sp.* NGR234 strain ANU280 DNA restricted with *Hin*dIII,
Lane 10 *R. sp.* NGR234 strain ANU280 DNA restricted with *Pst*I,
Lane 11 *R. sp.* NGR234 strain ANU280 DNA restricted with *Bam*HI,
Lane 12 λ DNA digested with *Hin*dIII.

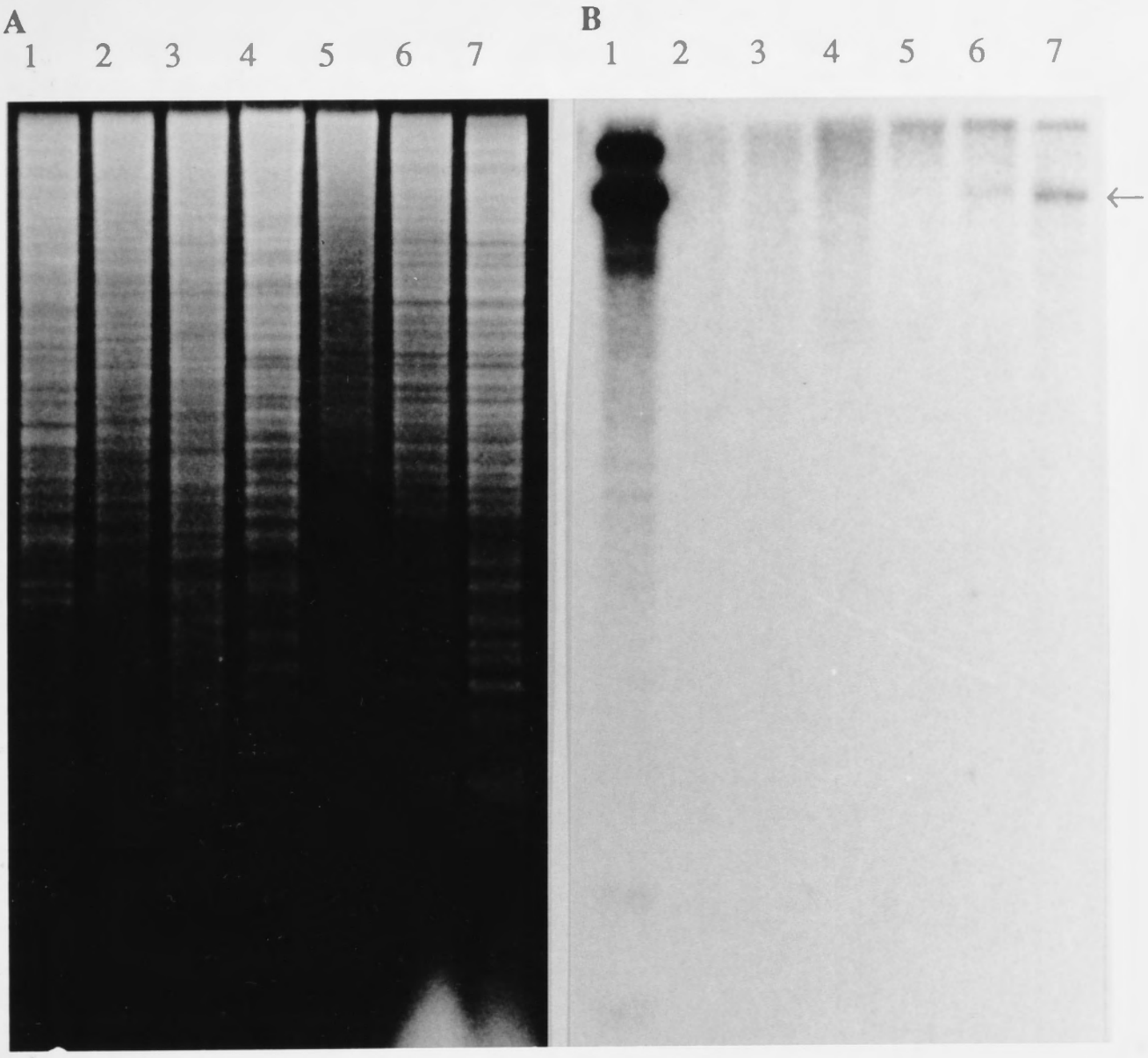


Fig. 6.3 Hybridization of *exo* DNA to *A. tumefaciens* genomic DNA. **(A)** Electrophoresis of *Hin*dIII restricted genomic DNA through a 0.8% agarose gel. **(B)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 10 kb *Bam*HI insert of pJG11. Hybridization conditions were not stringent (55°C, 0.9 M NaCl).

Lane 1 *R. sp.* NGR234 strain ANU280,
Lane 2 *B. japonicum* strain USDA110,
Lane 3 *B. parasponia* strain ANU289,
Lane 4 general cowpea *Bradyrhizobium* strain CB756,
Lane 5 degraded *A. tumefaciens* DNA,
Lane 6 *A. tumefaciens* strain A136 (pTi^-)*,
Lane 7 *A. tumefaciens* strain GMI9023 (pTi^-, pAt^-)*.

* A feint band of hybridizing DNA in lanes 6 and 7 is evident.

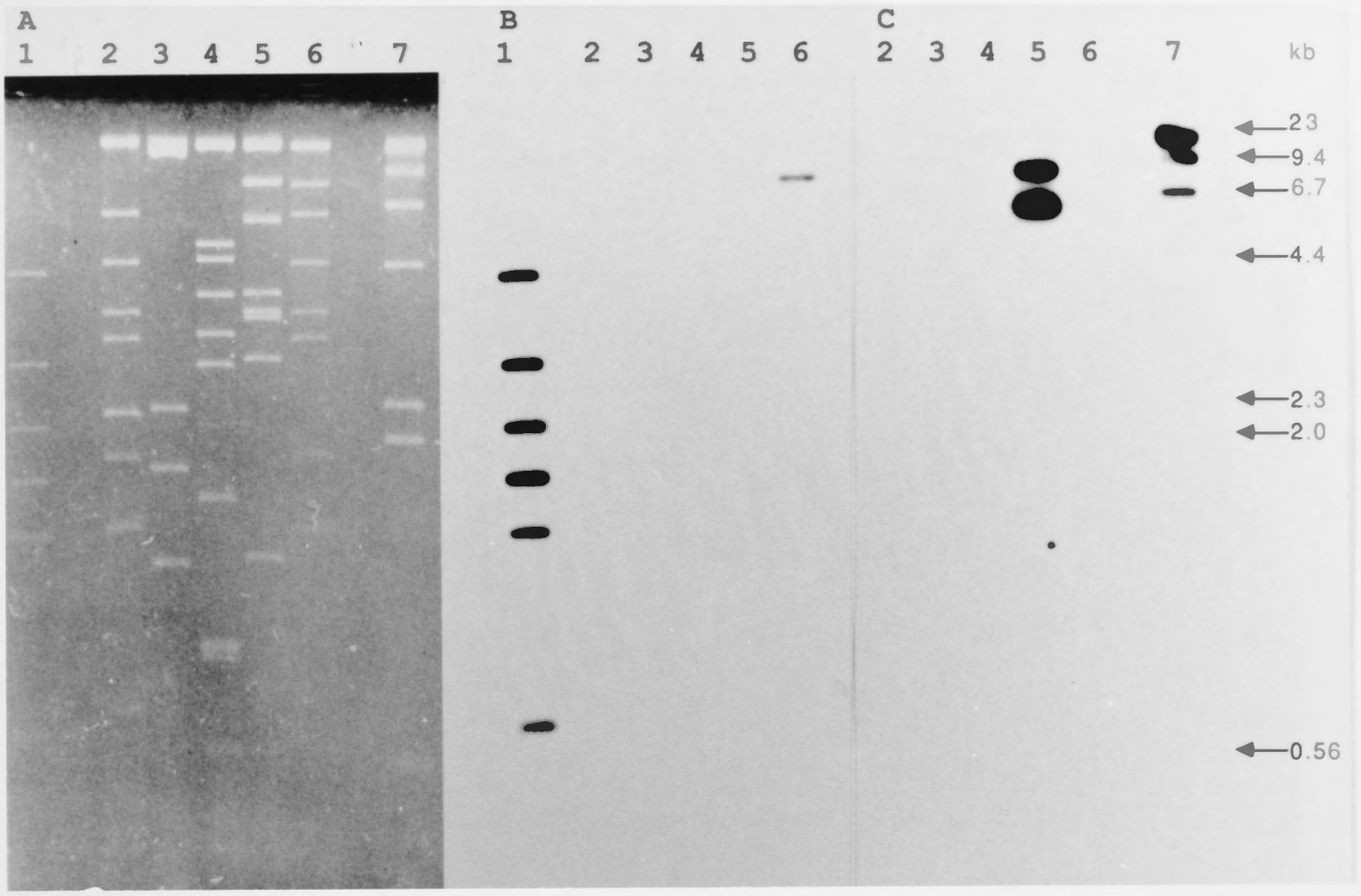


Fig. 6.4 Hybridization of cloned *exo* DNA from *R*. sp. NGR234 to the *R. meliloti exo* cosmids. **(A)** Electrophoresis of *Eco*RI restricted cosmid DNA through a 0.8% agarose gel. **(B)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 10 kb *Bam*HI insert of pJG11. **(C)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 9 kb *Bam*HI insert of pJG40. Hybridization conditions were stringent (65°C, 0.9 M NaCl).

Lane 1 pJG11 restricted with *Eco*RI,
Lane 2 pD2 restricted with *Eco*RI,
Lane 3 pD5 restricted with *Eco*RI,
Lane 4 pD15 restricted with *Eco*RI,
Lane 5 pD34 restricted with *Eco*RI,
Lane 6 pD56 restricted with *Eco*RI,
Lane 7 λ DNA restricted with *Hin*dIII.

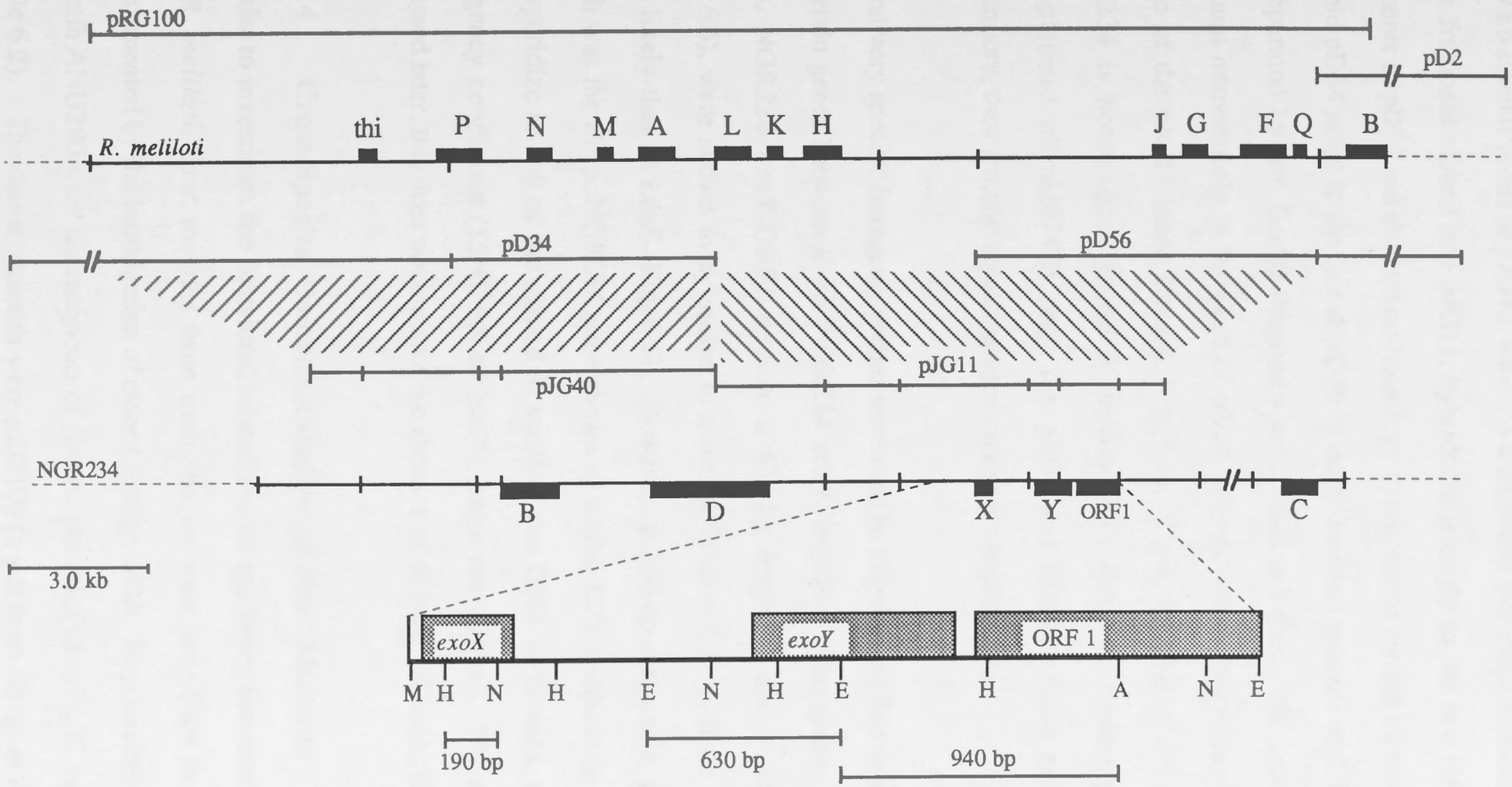


Fig. 6.5 Organization of *exo* genes in *R. meliloti* and *R.* sp. NGR234. Cross species hybridization studies revealed that the genetic organization between the two species is broadly similar. Cloned DNA and probe fragments mentioned in the text are summarized in this figure and sloped lines indicate regions of hybridization homology. *Eco*RI restriction sites are indicated by small vertical lines; and in the magnified region, restriction sites are: A, *Apa*I; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; N, *Nru*I.

fragment of pD56 and showed no homology to sequences carried by any other cosmids. The 9 kb *Bam*HI insert of pJG40, which is a fragment that maps immediately to the left of the fragment cloned into pJG11, hybridized strongly to the two right-most *Eco*RI fragments of pD34 and showed no homology to sequences carried by any other cosmids. Cosmid pD34 maps to the left of pD56 in the *R. meliloti* genome and the two cosmids are separated by two *Eco*RI fragments not cloned in either. The intervening *Eco*RI fragment immediately to the right of pD34, showed strong homology to the leftmost region of the pJG11 insert (lane 4, Fig. 6.2). Thus, the cloned *exo* DNA of *R.* sp. NGR234 is homologous to the *R. meliloti exo* DNA sequences present on the megaplasmid pRmeSU47b only (*ie.* pD34 and pD56), (Finan *et al.*, 1986) and furthermore, their genetic organization shows broad similarities.

Several very specific intragenic probes were used to examine the fine structure homology of certain genes between *R.* sp. NGR234 and *R. meliloti*. Two probes specific for the *R.* sp. NGR234 *exoY*-ORF1 operon, a 630 bp fragment and a 940 bp fragment (Fig. 6.5), were shown to hybridize to a region of cloned *R. meliloti* DNA, which is most likely that of *exoF* (Fig. 6.6). However, a 190 bp intragenic probe for *exoX*, which was the *R.* sp. NGR234 gene shown to inhibit EPS synthesis in *R. meliloti*, did not hybridize to any of the cloned *R. meliloti exo* DNA sequences, even under low stringency conditions (55°C, 0.9 M NaCl), (data not shown). For reasons that are discussed later, this does not indicate the absence of an *exoX* homolog in *R. meliloti*.

6.2.4 Cross-Species Complementation of Exo⁻ Mutants

In order to investigate the functional relatedness of *exo* genes between *R.* sp. NGR234 and *R. meliloti*, Exo⁻ mutants from each species were tested for their ability to be complemented by the introduction of cloned foreign DNA. Representative Exo⁻ mutants of strain ANU280 were the recipients of several plasmids carrying *R. meliloti exo* DNA (Table 6.2). The donor plasmids were pRG100 (a gift from Hangjun Zhan, University

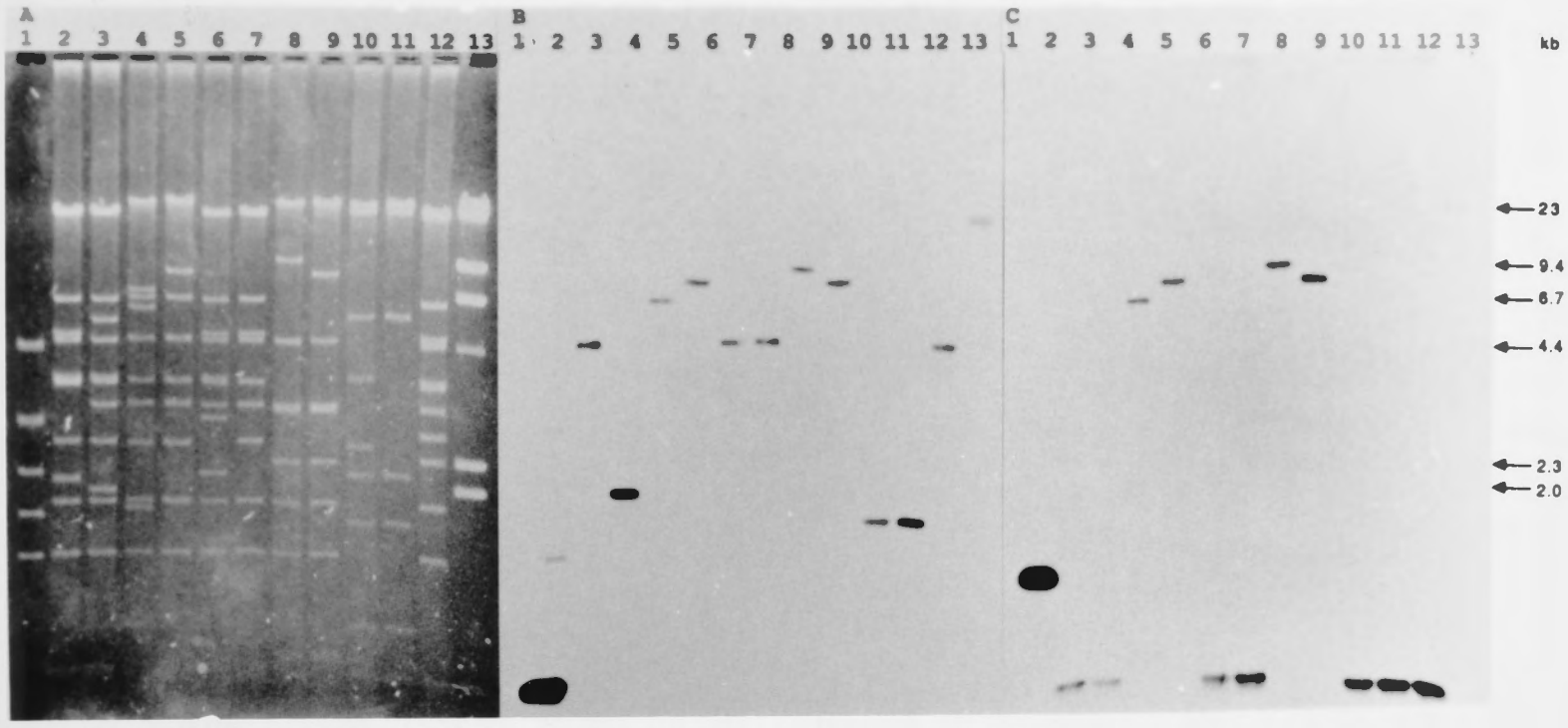


Fig. 6.6 Hybridization of *exoY* to pD56::Tn*5* cosmids. **(A)** Electrophoresis of restricted cosmid DNA through a 0.8% agarose gel. **(B)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 630 bp *Eco*RI fragment (Fig. 6.5). **(C)** Autoradiograph of a Southern blot of the gel displayed in panel A, that has been probed with ^{32}P-labelled 940 bp *Eco*RI-*Apa*I fragment (Fig. 6.5). Hybridization conditions were stringent (65°C, 0.9 M NaCl).

Lane 1 pJG11 restricted with *Eco*RI,
Lane 2 unidentified cosmid (Tn*5* maps to the 3 kb *Eco*RI fragment of pD56),
Lane 3 pD56-*exoG321*::Tn*5* restricted with *Eco*RI and *Hin*dIII,
Lane 4 pD56-*exoF306*::Tn*5* restricted with *Eco*RI and *Hpa*I,
Lane 5 pD56 restricted with *Eco*RI and *Hpa*I,
Lane 6 pD56-*exoF306*::Tn*5* restricted with *Eco*RI and *Hin*dIII,
Lane 7 pD56 restricted with *Eco*RI and *Hin*dIII,
Lane 8 pD56-*exoF306*::Tn*5* restricted with *Eco*RI and *Bam*HI,
Lane 9 pD56 restricted with *Eco*RI and *Bam*HI,
Lane 10 pD56-*exoF306*::Tn*5* restricted with *Hin*dIII and *Cla*I,
Lane 11 pD56 restricted with *Hin*dIII and *Cla*I,
Lane 12 pD56-*exoQ332*::Tn*5* restricted with *Eco*RI and *Hin*dIII,
Lane 13 λ DNA restricted with *Hin*dIII.

Table 6.2 Complementation of Exo⁻ mutants with *R. meliloti* cosmids

Strain and Class		Plasmid							
		pRG100	pD56	pD2	pD34	pD15	pD5	p347::Tn5[a]	R'3222
ANU2807	*exoY*	.	+/-[b]	-[c]	-	-	-	.[d]	+[e]
ANU2811	*exoY*	+	+/-	-	-	-	-	+/-	+
ANU2823	*exoY*	.	+/-	-	-	-	-	.	+
ANU2840	*exoY*	+	+/-	-	-	.	.	.	+
ANU2844	*exoY*	.	+/-	-	-	-	-	.	+
ANU2851	*exoY*	.	+/-	-	-	-	-	.	+
ANU2852	*exoY*	.	+/-	-	-	-	-	.	+
ANU2854	*exoY*	.	+/-	-	-	-	-	.	+
ANU2865	*exoY*	.	+/-	-	-	-	-	.	+
ANU2890	*exoY*	+	+/-	-	-	-	-	.	+
ANU2818	G	-	-	.	-	.	.	.	-
ANU2822	*exoC*	-	+	+	-	.	.	-	+
ANU2824	*exoC*	-	+	+	-	.	.	-	+
ANU2826	*exoB*	+	-	-	-	.	.	.	+
ANU2867	D	+	-	-	-	.	.	.	+
ANU2871	D	+	-	-	-	.	.	.	+
ANU280	wild-type	+	*[f]	+	+	+	+	+	+
Rm7013	*exoB*	.	+	+	-	.	.	.	+
Rm7031	*exoA*	.	-	-	+	.	.	.	+
Rm7055	*exoF*	.	+	-	-	.	.	.	+
Rm1021	wild-type	.	+	+	+	.	.	.	+

a Plasmid p347::Tn*5* is a derivative of pD56 with a Tn*5* within *exoB* and has the reference number 347 in Long *et al.* (1988).

b "+/-" indicates that the colony morphology of the transconjugant was only slightly more mucoid than the Exo⁻ morphology of the mutant.

c " - " indicates no difference from the Exo⁻ colony morphology of the mutant.

d "." indicates that the experiment was not done.

e " + " indicates that the colony morphology of the transconjugant was restored to that of the wild-type.

f The colony morphology (indicated by " * ") of this particular transconjugant was slightly less mucoid than the wild-type.

of Washington, see figure 6.5), pD56-*exoB347*::Tn*5* (a gift from Graham C. Walker, MIT), pD56, pD2, pD34, pD15, pD5 (Leigh *et al.*, 1985) and R'3222 (Chen *et al.*, 1988). Only those plasmids that carry DNA from the megaplasmid pRmeSU47b were able to correct the phenotypes of some Exo$^-$ mutants. Plasmid pRG100 restored an Exo$^+$ phenotype to the largest number of Exo$^-$ mutants: *exoY*::Tn*5* mutants and mutants of genetic classes B and D. The only mutants within the cluster of *exo* loci that were not corrected by pRG100, were those of genetic class C. Plasmid pD56 was able to confer the ability for *exoY*::Tn*5* transconjugants to form slightly mucoid colonies and interestingly ANU280(pD56) transconjugants produce less mucoid colonies than the wild-type; reasons for this are discussed later.

Mutants of genetic class C were fully restored to Exo$^+$ by the introduction of either pD56 or pD2. The DNA cloned on these two cosmids overlap and the only known *exo* gene to be carried by pD2 is the *R. meliloti exoB* gene. The *R.* sp. NGR234 genetic class C is the functional homolog of the *R. meliloti exoB* gene, because a derivative of pD56 with a Tn*5* insertion within the *exoB* locus, pD56-*exoB347*::Tn*5*, is unable to restore the Exo$^+$ phenotype to the genetic class C mutants. Furthermore, R'2822 (R'3222-C::Tn*5*) failed to restore an Exo$^+$ phenotype to *R. meliloti exoB* mutants, where as R'3222 does complement *exoB* mutants. The plasmid R'3222 also restored an Exo$^+$ phenotype to *R. meliloti* Exo$^-$ mutants at *exoA* and *exoF*; other *R. meliloti exo* mutants were not available to be tested.

Restoration of an Exo$^+$ phenotype by a foreign DNA fragment also resulted in the restoration of symbiotic effectiveness on the host plant for the recipient strain. Nitrogen-fixing nodules on *Leucaena* were formed by Exo$^+$ transconjugants of *R.* sp. NGR234 *exo* mutants carrying cloned *exo* DNA from *R. meliloti* (Table 6.3). Similarly, transconjugants *R. meliloti exoA*, *exoB* and *exoF* mutants carrying R'3222 were Exo$^+$ and able to form nitrogen-fixing nodules on alfalfa (Table 6.3). These results indicate

Table 6.3 Symbiotic phenotypes of Exo$^+$ transconjugants

Rhizobium construct	Genetic class	Exo phenotype	Symbiotic phenotype on plants *Leucaena*	alfalfa
ANU2822	C	-	calli	Nod$^-$
ANU2822(pD2)	C	+	Fix$^+$	Nod$^-$
ANU2822(pD56)	C	+	Fix$^+$	Nod$^-$
ANU2822(R'3222)	C	+	Fix$^+$	Nod$^-$
ANU2824	C	-	calli	Nod$^-$
ANU2824(pD2)	C	+	Fix$^+$	Nod$^-$
ANU2824(pD56)	C	+	Fix$^+$	Nod$^-$
ANU2824(R'3222)	C	+	Fix$^+$	Nod$^-$
Rm7013	*exoB*	-	Nod$^-$	calli
Rm7013(R'3222)	*exoB*	+	Nod$^-$	Fix$^+$
ANU2826	B	-	calli	Nod$^-$
ANU2826(pRG100)	B	+	Fix$^+$	Nod$^-$
ANU2867	D	-	calli	Nod$^-$
ANU2867(pRG100)	D	+	Fix$^+$	Nod$^-$
ANU2871	D	-	calli	Nod$^-$
ANU2871(pRG100)	D	+	Fix$^+$	Nod$^-$
Rm7031	*exoA*	-	Nod$^-$	calli
Rm7031(R'3222)	*exoA*	+	Nod$^-$	Fix$^+$
ANU2811	*exoY*	-	calli	Nod$^-$
ANU2811(pRG100)	*exoY*	+	Fix$^+$	Nod$^-$
ANU2890	*exoY*	-	calli	Nod$^-$
ANU2890(pRG100)	*exoY*	+	Fix$^+$	Nod$^-$
ANU2840	*exoY*	-	calli	Nod$^-$
ANU2840(pRG100)	*exoY*	+	Fix$^+$	Nod$^-$
Rm7055	*exoF*	-	Nod$^-$	calli
Rm7055(R'3222)	*exoF*	+	Nod$^-$	Fix$^+$
ANU280	wild-type	+	Fix$^+$	Nod$^-$
Rm1021	wild-type	+	Nod$^-$	Fix$^+$

At least eight plants were tested for each plant test. Nitrogen fixation ability was determined by acetylene reduction and plant growth. The term calli is used to describe the *Rhizobium* induced root growths that do not contain *Rhizobium* cells (sometimes referred to as empty or pseudonodules).

that many of the genes involved in EPS production for *R.* sp. NGR234 are active in the foreign background and able to substitute for *R. meliloti exo* genes and vice versa.

6.2.5 Restoration of EPS Synthesis by Deletion Mutants

The generation of *R.* sp. NGR234 Exo$^-$ mutant strains, that have deletions spanning most of the *exo* DNA region, are described in section 4.2.6 and will be referred to from here on as 616-d strains. The deletion has a terminus within *exoY* and extends a minimum of 16 kb upstream of *exoY*, effectively deleting the *exoY*-ORF1 operon, *exoX*, genetic classes B and D (Fig. 6.7). These deletion strains can not be complemented by the 10 kb *Bam*HI clone of pJG11, but they can be complemented by the larger *R. meliloti* cloned DNA fragment of pRG100 (Fig. 6.8). The exopolysaccharide produced by 616-d(pRG100) transconjugants is more likely to have a structure equivalent to *R. meliloti* EPS rather than *R.* sp. NGR234 EPS, because virtually every gene known to be involved in acidic EPS production has been deleted in the strain 616-d genome and has been replaced with the cluster of genes from *R. meliloti*, which are known to be responsible for the production of succinoglycan. This is also supported by ^{1}H-NMR analysis of the EPS produced by hybrid transconjugants, which was performed by Hangjun Zhan, Steven B. Levery and John A. Leigh (see chapter 6 acknowledgements).

The symbiotic properties of the Exo$^-$ 616-d deletion strains on *Leucaena* plants were no different to the symbiotic phenotypes of the Tn*5* generated Exo$^-$ mutants. Strain 616-d produced small white calli on the *Leucaena* roots that contained no bacteria and were Fix$^-$; they were indistinguishable from the calli generated by strain ANU2811 (Fig. 6.9 A and B). The 616-d(pRG100) transconjugant hybrid constructs had Exo$^+$ phenotypes with mucoid colony morphologies indistinguishable from that of wild-type *R.* sp. NGR234 strains (Fig. 6.8). These hybrid constructs were able to proceed further with nodule development than the Exo$^-$ deletion strains (Fig. 6.9 C), but symbiosis was still aborted

before the development of nitrogen-fixing nodules. The 616-d(pRG100) transconjugants were able to form small nodules on *Leucaena*, that contained bacteria, but were still Fix$^-$ (Fig. 6.10). Therefore, simply restoring an Exo$^+$ phenotype is not sufficient for symbiotic restoration and this is most likely because the EPS produced by the hybrids was heterologous to the EPS associated with the wild-type *R.* sp. NGR234. There was no response by alfalfa plants to the inoculation of Exo$^+$ 616-d(pRG100) transconjugants on the roots of alfalfa seedlings. The hybrids failed to induce any plant cell differentiation, nor was there any curling or deformation of alfalfa root hairs.

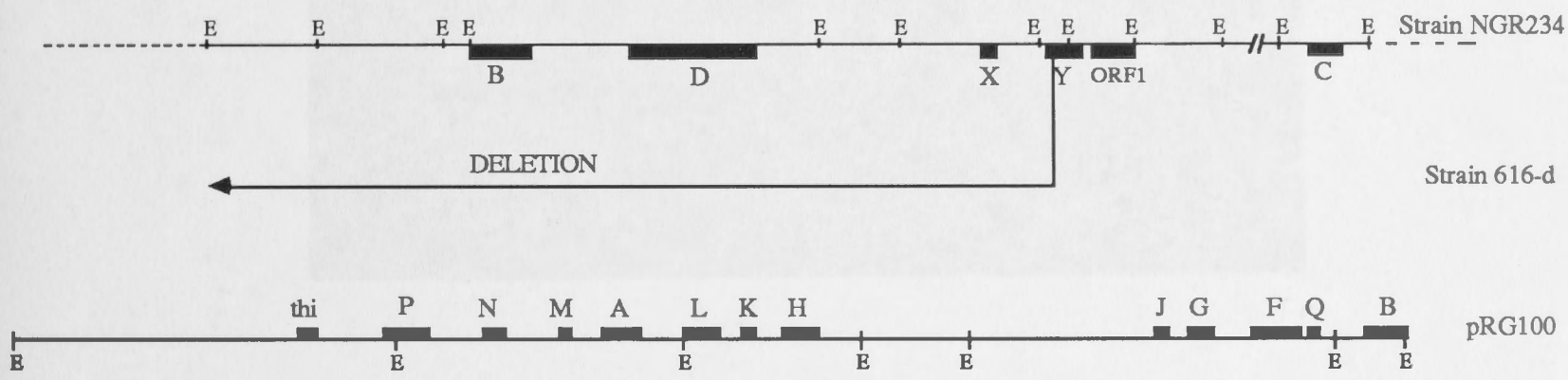


Fig. 6.7 Physical description of 616-d(pRG100) transconjugants. The region of *exo* DNA deleted from the *R.* sp. NGR234 genome is indicated and the genetic map of pRG100 carrying *R. meliloti exo* DNA is shown. Both genetic regions are represented against an *Eco*RI (E) restriction map.

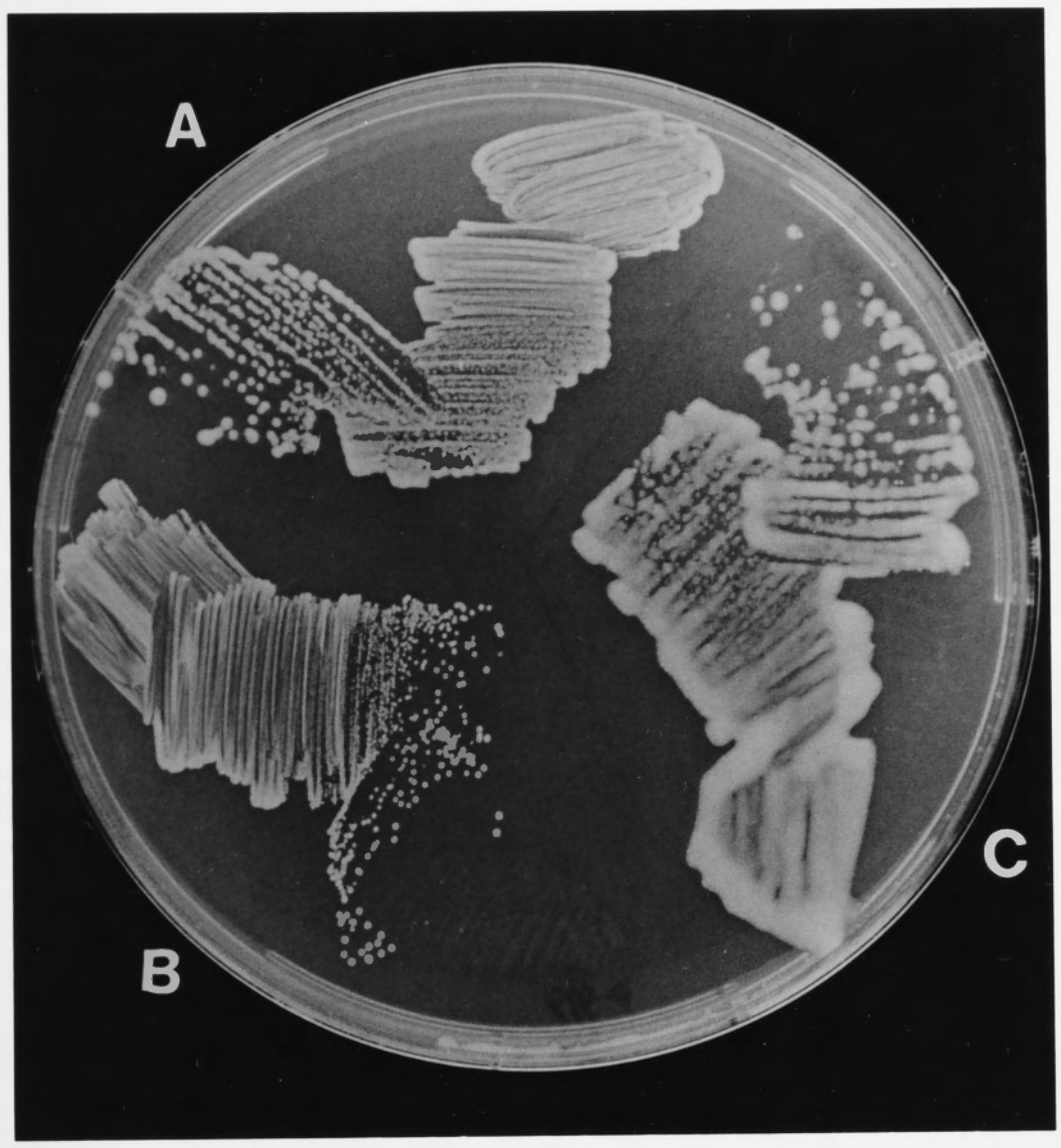


Fig. 6.8 Comparison of colony morphologies. **(A)** Exo$^+$ wild-type strain ANU280, **(B)** Exo$^-$ deletion strain 616-d and **(C)** Exo$^+$ transconjugant strain 616-d(pRG100). Strains were cultured on solid BMM media for four days.

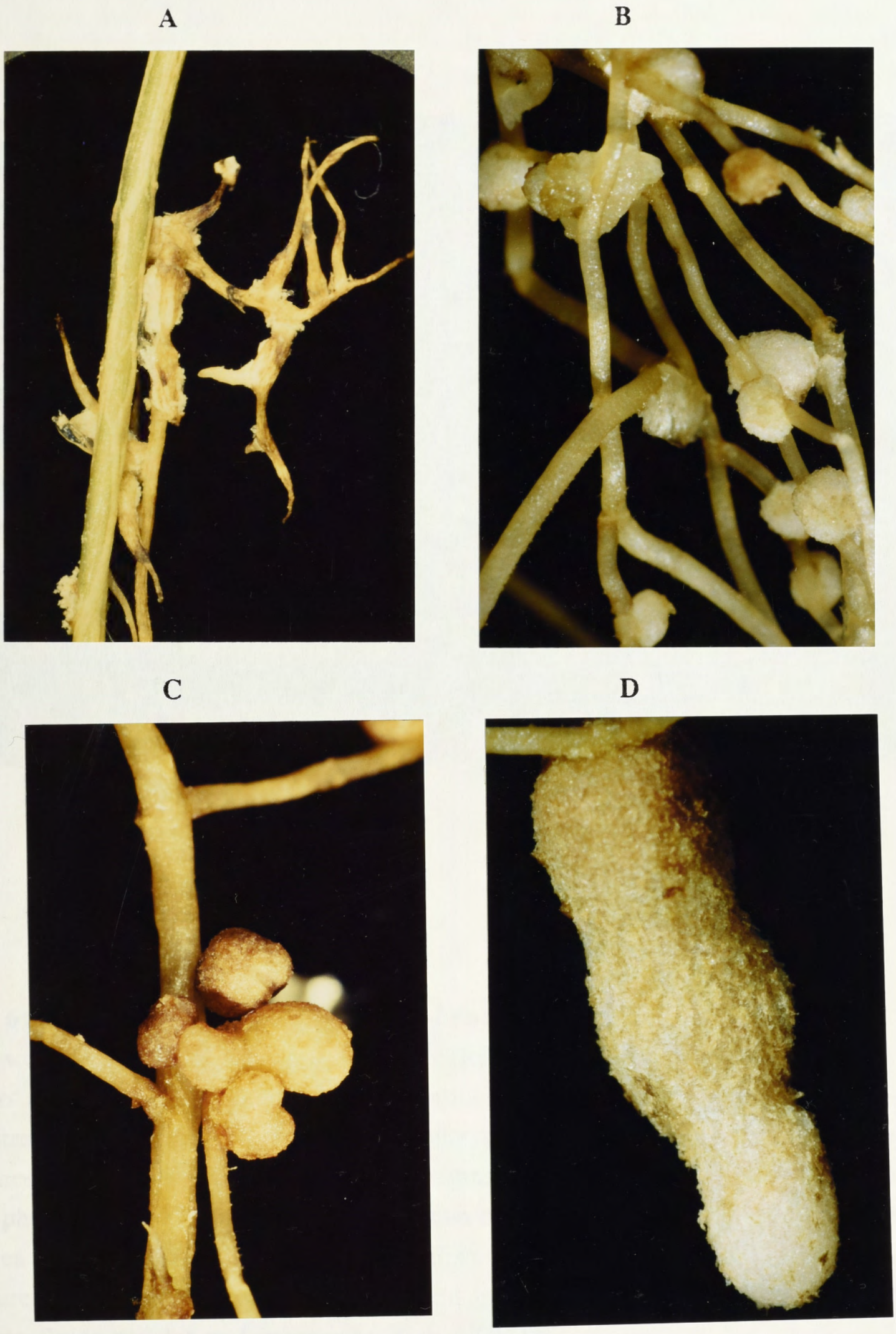


Fig. 6.9 Nodule and calli structures on *Leucaena*. Plants were inoculated with **(A)** The *exoY*::Tn*5* mutant strain ANU2811, forms calli; **(B)** deletion strain 616-d, forms calli; **(C)** transconjugant strain 616-d(pRG100), forms Fix^- nodules; **(D)** wild-type strain ANU280, forms Fix^+ nodules. The structures were photographed from five week old *Leucaena* plants.

Fig. 6.10 Comparative growth of *Leucaena* plants. Photographed after seven weeks. The plants were inoculated with: (left) the deletion strain 616-d, which has an Exo^- phenotype and as a consequence does not nodulate *Leucaena*; (center) the wild-type strain ANU280, which has an Exo^+ phenotype and forms nitrogen fixing nodules on *Leucaena*; and (right) the hybrid transconjugant strain 616-d(pRG100), which has an Exo^+ phenotype, but the EPS synthesized is that of *R. meliloti* (succinoglycan) and the nodules resulting on *Leucaena* plants are Fix^-. The result suggests that the actual structure of the acidic EPS molecule is important during infection for the establishment of nitrogen-fixing nodules on *Leucaena* by *R.* sp. NGR234.

6.3 DISCUSSION

The general system for EPS synthesis and regulation is probably the same in all fast-growing *Rhizobium* species and other soil bacteria as well. In this chapter it was demonstrated that, multiple copies of the *exoX* gene from *R.* sp. NGR234 could effectively inhibit EPS synthesis in *R. fredii* and *R. meliloti* (*R. l.* bv. *phaseoli* and *R. l.* bv. *viciae* were not tested). Similarly, a gene in *R. meliloti* strain SU47, which is believed to be the functional equivalent to *exoX*, was able to inhibit EPS production in *R. meliloti*, *R. l.* bv. *phaseoli* and *R. l.* bv. *viciae* when present in high gene dosage and in the absence of *exoF* (personal communication from Hangjun Zhan). The DNA sequence of the *R. meliloti exoX* gene is 61% homologous to the sequence of *exoX* in *R.* sp. NGR234, confirming that they are the same gene (personal communication from Jason W. Reed, M.I.T.). The gene equivalent to *exoX* in *R. l.* bv. *phaseoli* is *psi* (Borthakur *et al.*, 1985). Although *psi* and *exoX* are only 33% homologous at the DNA sequence level, they do have a short, highly conserved region of peptide homology, a high level of secondary structure homology and they have very similar phenotypes (see chapter 4). In addition, *A. tumefaciens* has a gene, *psdA* (Kamoun *et al.*, 1989), that also inhibits EPS synthesis.

Role of *nodD2* in the regulation of EPS synthesis

EPS production in *R.* sp. NGR234 can also be inhibited by the introduction of a plasmid carrying the *R. fredii* gene termed *nodD2* (Appelbaum *et al.*, 1988). Multiple copies of *nodD2* inhibits EPS in a manner that appears identical to that obtained from the introduction of multiple copies of *exoX*. The *nodD2* gene encodes a polypeptide of 313 amino acids that is 69% related to the polypeptide encoded by *nodD1* of *R. fredii* and there is absolutely no DNA or peptide homology between any regions of *nodD2* and the *exoX* gene or polypeptide. The *nodD2* gene does not regulate the *nodABC* operon in a manner generally associated with *nodD*, but instead its only known phenotype is inhibition of EPS production. The total lack of homology to *exoX* indicate that *nodD2* is

unlikely to repress EPS synthesis by the same mechanism as *exoX*, but it may very possibly inhibit EPS synthesis within the same pathway as *exoX*. Inhibition of EPS production by *exoX* is dependent on a lower relative gene dosage of *exoY* (chapter 4), and in the wild-type situation it is most likely the relative levels of expression between *exoX* and *exoY* that governs EPS regulation. Analysis of the *exoY* promoter shows sequences that are related to *nod*-box sequences. It is possible that *nodD2* regulates transcription of *exoY* via these *nod*-box sequences, in a manner analogous to the regulation of *nodABC* via *nodD1*. The *nodD2* gene may be an environment sensing gene affecting EPS synthesis, just as *nodD1* senses plant signals prior to the induction of *nod* operons. Perhaps in the wild-type situation, the *nodD2* gene product may bind to this promoter in the presence of environmental stimuli or plant signals and repress its transcription; with the net result being the inhibition of EPS synthesis in a manner similar to that caused by the absence of *exoY*. The repression of *exoY* for the specific purpose of inhibiting EPS synthesis, may occur during the later stage of nodule development, so that carbon can be allocated for nitrogenase activity rather than used in EPS production. *R*. sp. NGR234 very likely has its own *nodD2* homolog, because a reiterated DNA sequence homologous to its own *nodD* gene was detected by hybridization to genomic DNA (personal communication from Jacek Plazinski, RSBS, ANU.). Furthermore, the putative *R*. sp. NGR234 *nodD2* gene does not have any known biological functions that can substitute for those of *nodD*, because Tn*5* mutations within *nodD* are completely Nod$^-$ on all host plants tested (Morrison *et al*., 1984).

Functional homology of *exo* genes in rhizobia

Further evidence that *exo* genes are related among the fast-growing rhizobia and other soil bacteria, come from cross-species hybridization and complementation experiments. This chapter presented evidence that *exo* DNA sequences between *R*. sp. NGR234, *R. meliloti* and *R. fredii* are highly related and a lower level of relatedness was detected between *R*. sp. NGR234 and *A. tumefaciens*. In addition, some genes were

functionally interchangeable between *R.* sp NGR234 and *R. meliloti* and it was reported by Borthakur *et al.* (1986) that a cloned fragment of *Xanthomonas campestris* DNA was able to correct the Exo$^-$ and symbiotic phenotypes of *pss2* (homologous to *exoY*, chapter 4) mutants of *R. l.* bv. *phaseoli* and *R. l.* bv. *viciae*.

The cluster of *exo* genes in *R.* sp. NGR234 has a very similar gene order to the cluster of *R. meliloti exo* genes on the megaplasmid pRmeSU47b. Cloned *R.* sp. NGR234 DNA was used to probe cloned *R. meliloti* DNA and the hybridizing fragments between the two species followed the same sequential order. Furthermore, overlapping cloned *R. meliloti* DNA functionally complemented *R.* sp. NGR234 Exo$^-$ mutants in the same sequential genetic order as that observed by DNA homology.

The *R.* sp. NGR234 plasmid R'3222 was able to functionally complement *R. meliloti* mutants at loci *exoA*, *exoB* and *exoF*, such that the Exo$^+$ transconjugants were able to form nitrogen-fixing nodules on alfalfa. A similar result was also obtained for mutants at loci *exoL*, *exoM* and *exoP* (personal communication from Hangjun Zhan). Staining with calcofluor and proton NMR spectroscopy confirmed that the EPS produced by the transconjugants contained *R. meliloti* succinoglycan (personal communication from Hangjun Zhan, Steven Levery and John Leigh).

Mutations in *R.* sp. NGR234 *exo* loci could also be functionally complemented by cloned *R. meliloti* DNA. Cosmids pD2 and pD56 complemented the genetic class C mutants and it was confirmed that this locus was equivalent to the *R. meliloti exoB*. Cosmid pD56 had two interesting complementation properties: i) *exoY*::Tn*5*(pD56) transconjugants were partially corrected such that they produced low levels of EPS, and ii) ANU280(pD56) transconjugants produced less EPS than strain ANU280 cells. This latter observation was also observed when pD56 was transferred into wild-type *A. tumefaciens* (Kamoun *et al.*, 1989). These two apparently conflicting properties can

be easily reconciled when one considers that pD56 carries equivalents to the two genes *exoX* and *exoY*. The *exoX* gene is the inhibitor of EPS synthesis and is essentially fully functional in all three bacterial backgrounds. The ability of pD56 to partially complement *exoY*::Tn*5* mutants, indicates that the donor "*exoY*" gene is obviously functional in the *R*. sp. NGR234 background, but with reduced efficiency and it is perhaps not functional at all in the *A. tumefaciens* background. Therefore, the level of biological activity associated with *exoY* is more dependent than *exoX,* on the bacterial background. The *R. meliloti* gene equivalent to *exoY* is termed *exoF* and is carried by pD56. An intragenic probe for *exoY* was found to hybridize strongly to a 0.75 kb *Hin*dIII fragment, which contains insertion sites for *exoF* mutations. Furthermore, 79% DNA sequence homology exists between *exoY* and *exoF* (personal communication from Jason W. Reed).

Another *R. meliloti* cosmid, pRG100, was able to functionally complement *R*. sp. NGR234 mutants at genetic loci B, D, and *exoY*. The complementation results, genetic maps of overlapping clones and ^{1}H-NMR spectroscopy results (personally communicated by Hangjun Zhan, Steven Levery and John Leigh) are summarized in figure 6.11. While most of the *R*. sp. NGR234 *exo* genes have functional counterparts in the *R. meliloti exo* region, it appears that genetic group B is an exception (experiment conducted by Hangjun Zhan). Plasmid pEX154 shares a common left-end terminus with pRG100 (Fig. 6.11) and it can only functionally complement genetic class D mutants and not those in genetic class B, where as plasmid pRG100 can complement both genetic classes. Mutations for genetic class B map between the genetic class D mutation sites and the common left-end terminus.

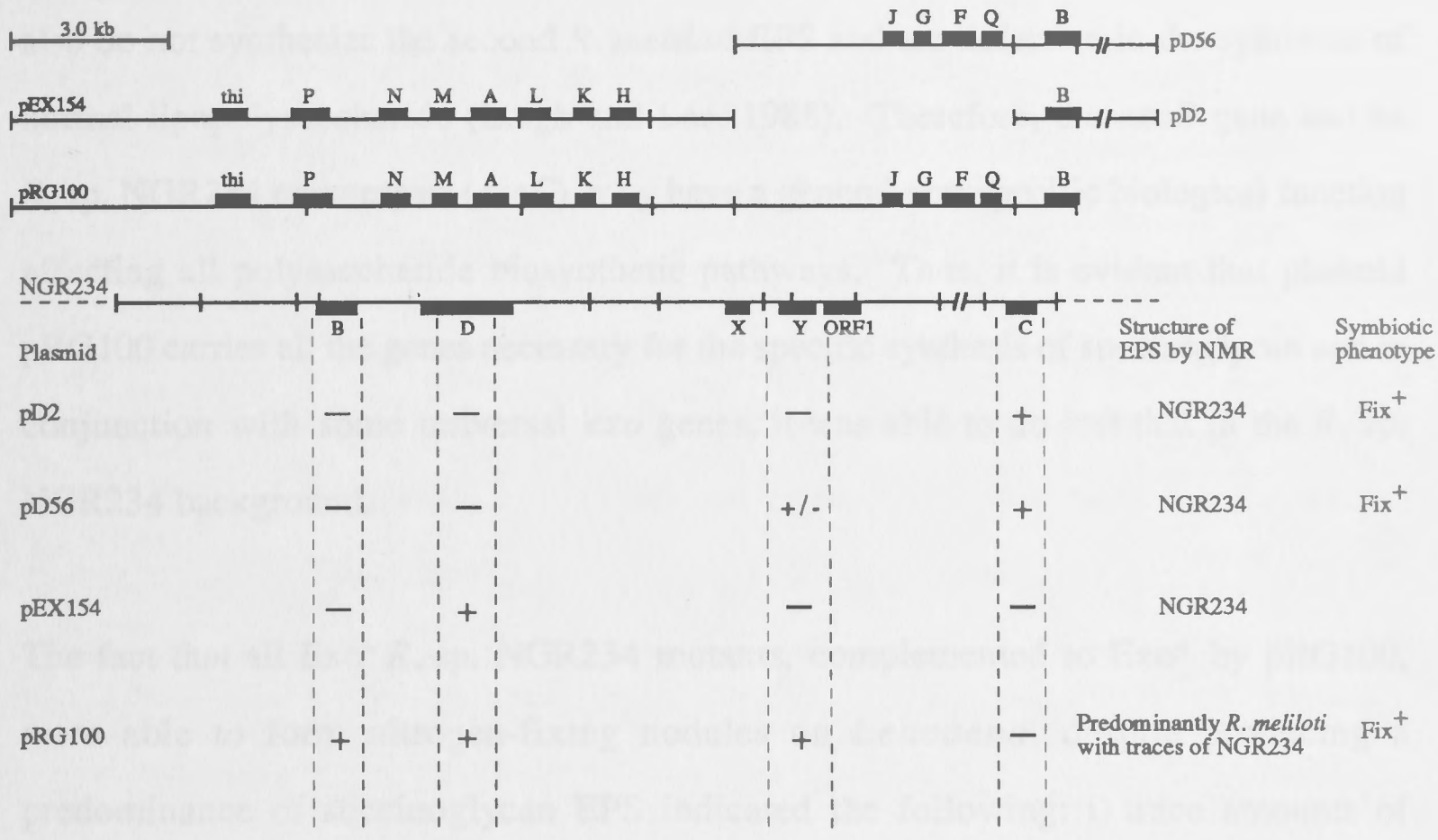


Fig. 6.11 Complementation of *R.* sp. NGR234 *exo* mutants with cloned *R. meliloti exo* DNA. The genetic map of a set of overlapping *R. meliloti* clones are shown above a genetic map of the clustered *exo* genes from *R.* sp NGR234. The complementation results for the *R. meliloti* cosmids in various *R.* sp. NGR234 mutant backgrounds is presented as a table. These results were collected from experiments done in collaboration with Hangjun Zhan, Steven Levery and John Leigh, particularly the ^{1}H-NMR data and the complementation results involving pEX154. The symbol +/- indicates that the colony morphology was only slightly more mucoid than the Exo$^-$ colony of the mutant.

All of the Exo$^+$ hybrid transconjugants were able to form nitrogen-fixing nodules on *Leucaena*. However, interesting observations about the EPS structures were made by ^{1}H-NMR spectroscopy (personally communicated by Hangjun Zhan, Steven Levery and John Leigh); Exo$^+$ transconjugants carrying pD2, pD56 or pEX154 all synthesized strain NGR234-like EPS, while Exo$^+$ transconjugants carrying pRG100 synthesized *R. meliloti* succinoglycan-like EPS and in some cases traces of NGR234-like EPS were also detected. Plasmid pRG100 encompasses the entire cluster of *R. meliloti exo* genes with the exception of *exoB*. Apart from not synthesizing succinoglycan, *exoB* mutants

also do not synthesize the second *R. meliloti* EPS and are defective in the synthesis of normal lipopolysaccharide (Leigh and Lee, 1988). Therefore, the *exoB* gene and its *R.* sp. NGR234 counterpart (*exoC*), may have a general non-specific biological function affecting all polysaccharide biosynthetic pathways. Thus, it is evident that plasmid pRG100 carries all the genes necessary for the specific synthesis of succinoglycan and in conjunction with some universal *exo* genes, it was able to do just that in the *R.* sp. NGR234 background.

The fact that all Exo$^-$ *R.* sp. NGR234 mutants, complemented to Exo$^+$ by pRG100, were able to form nitrogen-fixing nodules on *Leucaena*, despite producing a predominance of succinoglycan EPS indicated the following: i) trace amounts of NGR234-like EPS were also being synthesized and this was sufficient for symbiosis, and ii) the presence of succinoglycan was not interfering with nodule development. The reason why the transconjugants produced a predominance of succinoglycan is probably due to the fact that the foreign *exo* genes function coordinately and can more efficiently assemble precursors into succinoglycan rather than the EPS structure of the host cell. Also, since the foreign genes are located on a plasmid, this gives them a copy number advantage in a competition where precursors may be limiting. However, clearly some degeneracy exists within the donor genes, because the mutant recipient background is able to utilize the respective foreign gene for the synthesis of its own EPS, albeit at a low level.

R. sp. NGR234 deletion mutants (strain 616-d) had lost the entire cluster of *exo* genes with the exception of *exoC* and could be complemented to Exo$^+$ by the introduction of pRG100. Strain 616-d was Exo$^-$ and produced calli on the roots of *Leucaena*, that resembled the calli induced by Exo$^-$ mutants generated by Tn*5*. This indicated that although the deletion was extensive, it did not eliminate the ability of the *Rhizobium* to elicit cortical cell division and other early nodulation responses. Therefore, if signal

molecules such as the acylated tetrasaccharide molecule, which is secreted by *R. meliloti* and elicits early nodulation activity on alfalfa (Lerouge *et al.*, 1990), are present in the *R.* sp. NGR234-*Leucaena* symbiotic system, then they are still synthesized by strain 616-d deletion mutants.

Strain 616-d(pRG100) transconjugants were Exo$^+$ with colony morphologies that were indistinguishable from those of the wild-type strain ANU280. These transconjugants, despite being Exo$^+$, were unable to form nitrogen-fixing nodules on *Leucaena*. The EPS structure generated by these transconjugants was almost certainly succinoglycan, because it was established that pRG100 has all the necessary *exo* genes and the deletion has removed the entire cluster of specific NGR234 *exo* genes. Therefore, the absence of any NGR234-like EPS resulted in an inability to form nitrogen-fixing nodules on its host plant. The presence of a heterologous EPS, despite it being synthesized *in situ*, was unable to substitute for the wild-type EPS. These results are similar in principle, to those obtained by Djordjevic *et al.* (1987); wherein *exoY*::Tn*5* mutants of *R.* sp. NGR234 could form nitrogen-fixing nodules on *Leucaena* only when coinoculated with purified homologous NGR234 EPS or with strain ANU265 (pSym$^-$ derivative of *R.* sp. NGR234 and Exo$^+$), but not when coinoculated with heterologous *R. l.* bv. *trifolii* EPS or strain ANU845 (pSym$^-$ derivative of *R. l.* bv. *trifolii* and Exo$^+$). Small nodules that do not fix nitrogen resulted from a mixed inoculation involving *R.* sp. NGR234 *exoY*::Tn*5* mutants and *R. l.* bv. *trifolii* strain ANU845 (Djordjevic *et al.*, 1987). The same result was observed when 616-d(pRG100) transconjugant strains were inoculated onto *Leucaena* seedlings. The results clearly indicate that homologous EPS is necessary for the formation nitrogen-fixing nodules, but the synthesis or presence of heterologous EPS does allow nodule development to proceed a little further beyond that of the callus structures generated by the Exo$^-$ mutants.

The transconjugant strain 616-d(pRG100) did not elicit any response on the root systems of alfalfa plants. This is not surprising, since the symbiotic plasmid of this transconjugant strain will not have the *R. meliloti* host range genes, such as *nodH* and *nodQ*. The *nodH* and *nodQ* genes together with *nodABC* have all been shown to be involved in the synthesis of extracellular Nod signals, that are specific for alfalfa and cause root hair deformation and cortical cell division on alfalfa roots (Banfalvi and Kondorosi, 1989; Faucher *et al.*, 1989; Lerouge *et al.*, 1990).

Publication

Some of the information presented in this chapter was my contribution to the publication:-

Zhan, H., J. X. Gray, S. B. Levery, B. G. Rolfe, and J. A. Leigh. Functional and evolutionary relatedness of genes for exopolysaccharide synthesis in *Rhizobium meliloti* and *Rhizobium* sp. strain NGR234. *Journal of Bacteriology*. **172**:5245–5253.

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CHAPTER SEVEN
General Discussion

7.1 Evidence for an EPS Biosynthetic Complex That Is Regulated by One of the Subunits

The current knowledge on EPS biosynthesis of *Rhizobium* bacteria comes largely from the genetic studies of three different fast-growing *Rhizobium* species over the last five years, *R. l.* bv. *phaseoli*, *R. meliloti* strain SU47 and *R.* sp. strain NGR234. *R. meliloti* has a cluster of 13 genes on the second megaplasmid, pRmeSU47b (Long *et al.*, 1988), and several other genes located on the chromosome for EPS synthesis (*exo*) (Finan *et al.*, 1986). Similarly, a cluster of *exo* genes has been identified in *R.* sp. NGR234 (chapter 3) and most of these are functionally homologous to the cluster of *R. meliloti exo* genes (chapter 6). A second type of EPS that is synthesized by *R. meliloti* involves a further six genes on pRmeSU47b and another on the chromosome (Glazebrook and Walker, 1989; Zhan *et al.*, 1989).

The first inhibitor of EPS synthesis to be characterized in some detail was *psi* from *R. l.* bv. *phaseoli* (Borthakur, *et al.*, 1985, Borthakur and Johnston, 1987). Genes homologous to *psi* have since been discovered in several other bacterial species (Fig. 7.1): *psdA* in *A. tumefaciens* (Kamoun *et al.*, 1989), *exoX* in *R.* sp. NGR234 (chapter 4) and *exoX* in *R. meliloti* (Zhan and Leigh, 1990). The common characteristic of these genes is that when they are cloned on multicopy plasmids and transferred into their respective wild-type (Exo^+) *Rhizobium* strains, the transconjugants have Exo^- phenotypes. Mutants that carry Tn*5* insertions within the genomic copies of *psi* (Borthakur *et al.*, 1985), *exoX* (Zhan and Leigh, 1990; Keller *et al.*, 1990; Jason W. Reed, M.I.T., personal communication) or *psdA* (Kamoun *et al.*, 1989) produce either normal or increased levels of EPS. Thus, there is no absolute requirement for *exoX*-like genes in EPS production. DNA sequence analysis of *exoX* (from *R.* sp. NGR234) and

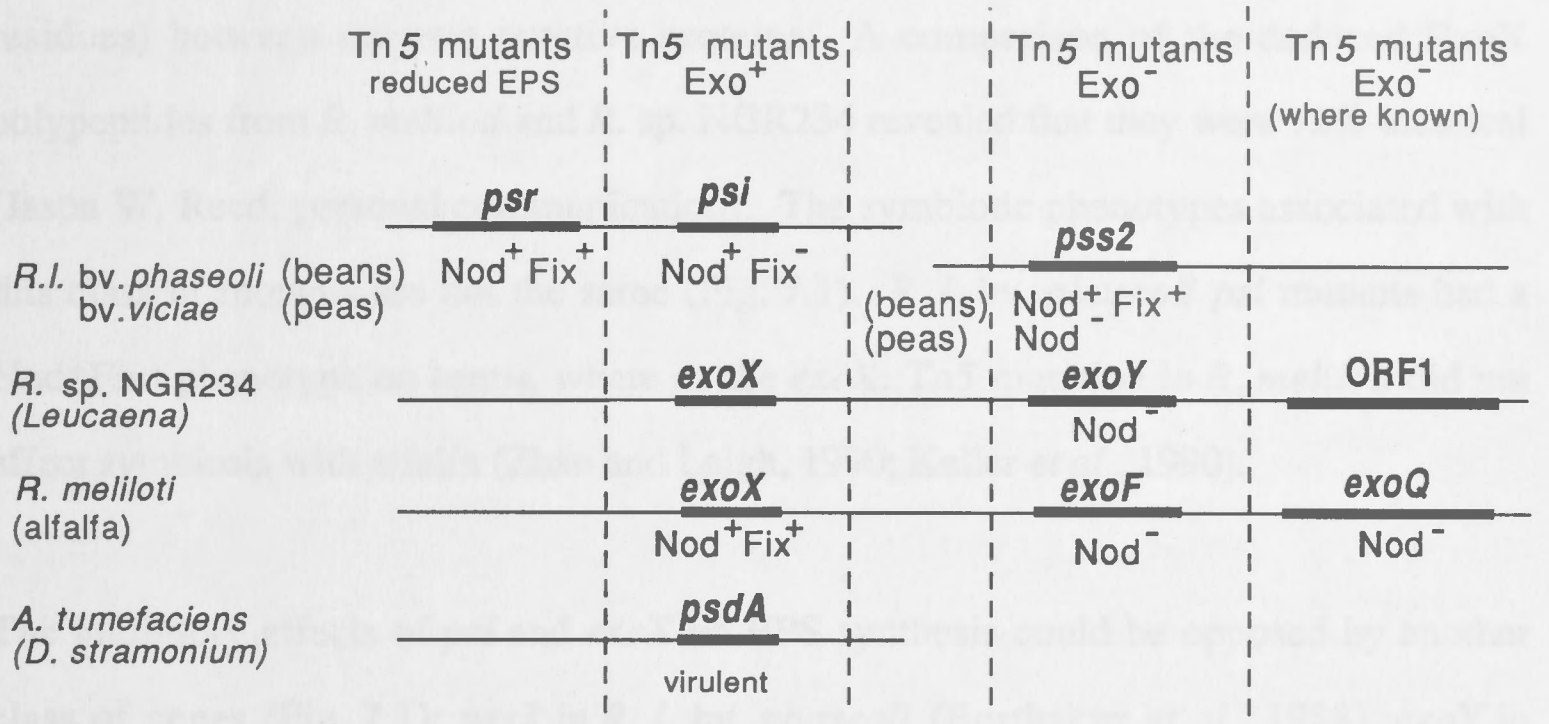

Fig. 7.1 Summary of the phenotypic properties of different regulatory and EPS synthesis mutants. The related genes are grouped into columns with their particular EPS phenotype (Exo), nodulation (Nod) and fixation (Fix) phenotypes (when known) also shown. Nod$^-$ for *exoY, exoF* and *exoQ* are either calli or pseudo nodules without bacteria. Genes belonging to the same bacterial species have been grouped into rows with the name of the species along the left hand side: *R. l.* bv. *phaseoli* (symbiosis with beans), *R. l.* bv. *viciae* (symbiosis with peas), *R.* sp. NGR234 (symbiosis with *Leucaena*), *A. tumefaciens* (tumors on *Datura stramonium*). Genes are depicted by thick lines and their linkage on the same DNA molecule is represented by thin unbroken horizontal lines, while unlinked genes (in the case of *R. leguminosarum* biovars) are represented by separate horizontal lines.

psi showed that the two genes encode proteins of similar size, 96 and 86 amino acids respectively. In addition, the hydrophobicity plots are very similar, but at the primary sequence level, there is only an 18 amino acid domain of homology (with 10 identical residues) between the two putative proteins. A comparison of the deduced ExoX polypeptides from *R. meliloti* and *R.* sp. NGR234 revealed that they were 72% identical (Jason W. Reed, personal communication). The symbiotic phenotypes associated with this class of mutants are not the same (Fig. 7.1). *R. l.* bv. *phaseoli psi* mutants had a $Nod^{+}Fix^{-}$ phenotype on beans, where as the *exoX*::Tn*5* mutation in *R. meliloti* did not affect symbiosis with alfalfa (Zhan and Leigh, 1990; Keller *et al.*, 1990).

The inhibitory effects of *psi* and *exoX* on EPS synthesis could be opposed by another class of genes (Fig. 7.1): *pss2* in *R. l.* bv. *phaseoli* (Borthakur *et al.*, 1988), *exoY* in *R.* sp. NGR234 (chapter 4) and *exoF* in *R. meliloti* (Zhan and Leigh, 1990). One common property of these genes (*pss2*/*exoY*/*exoF*) is that the inhibition of EPS caused by multicopy *psi*/*exoX* is overcome when the copy number of their counterpart (*pss2*/*exoY*/*exoF*) is at a comparable level. For example, normal EPS production occurs in *R.* sp. NGR234 transconjugants when both *exoY* and *exoX* are carried together on a plasmid, but inhibition of EPS production occurs when only *exoX* is plasmid borne. Mutations within the *pss2*, *exoY*, and *exoF* loci all result in an Exo^{-} phenotype. Only in the case of the *exoY* mutants has it been established that no oligosaccharide repeat unit is made (Djordjevic *et al.*, 1987b) and presumably this is also true for the other mutated loci. The nucleotide sequences for *exoY* and *pss2* have been determined and they are clearly homologs. ExoY (226 amino acids) and Pss2 (200 amino acids) are 52% similar with 32% of the homology due to exact matches and their hydrophobicity plots are virtually superimposable (chapter 4). A similar comparison by Keller *et al.* (1990) shows the homology between *exoF* and *pss2* and the polypeptide sequences of *exoF* and *exoY* are 79% identical (J. W. Reed, personal communication). In addition to the loss of exopolysaccharide synthesis, *Rhizobium* strains mutated in the *pss2*, *exoY* or *exoF* genes

are unable to nodulate their respective hosts (peas, *Leucaena* or alfalfa respectively). These hosts are all examples of legumes that develop an indeterminate nodule. In marked contrast, the Exo$^-$ *R. l* bv. *phaseoli pss2*::Tn*5* mutants still form nitrogen-fixing nodules on *Phaseolus* beans, which use a different ontogeny for the growth of a determinate nodule.

The *exoY* and *pss2* genes do not inhibit transcription of *exoX* (chapter 5) and *psi* (Borthakur *et al.*, 1988) respectively. In *R. l.* bv. *phaseoli*, transcription of *psi* is repressed by a third gene, *psr* (Borthakur and Johnston, 1987). However, mutations within *psr* results in a reduction, but not complete loss of EPS production. This suggests that the role played by *psr* is not as great as *pss2*, with regard to maintaining balanced EPS production. Furthermore, *psr* mutants of *R. l.* bv *phaseoli* are still fully symbioticly effective on beans (Borthakur and Johnston, 1987).

A model of the proposed processing complex for EPS synthesis and its regulation is shown in Fig. 7.2. The sequence analysis of *exoX, psi, exoY* and *pss2* demonstrates that their encoded proteins all have significant stretches of hydrophobic amino acids and thus argues that they may form a post-translational complex associated with the membrane. The genes *psi* and *exoX* are not required for EPS biosynthesis, yet *pss2, exoY* and *exoF* are essential. Thus, it is possible that the gene products from *pss2/exoY/exoF* form the central component of an EPS production complex that is regulated by their counterparts, *psi* or *exoX*. The gene products of *psi* and *exoX* would effect their regulation by reversibly binding to the complex (Fig. 7.2). For example, when ExoX is bound to the ExoY complex, no EPS production would occur and EPS production would resume again when ExoX disassociates from the complex. This model is supported by the fact that elevated copies of *exoX* would presumably result in the greater occurrence of ExoX inhibitor subunits and consequently more opportunities for the complex to be in a repressed state, resulting in the observed Exo$^-$ phenotype. When

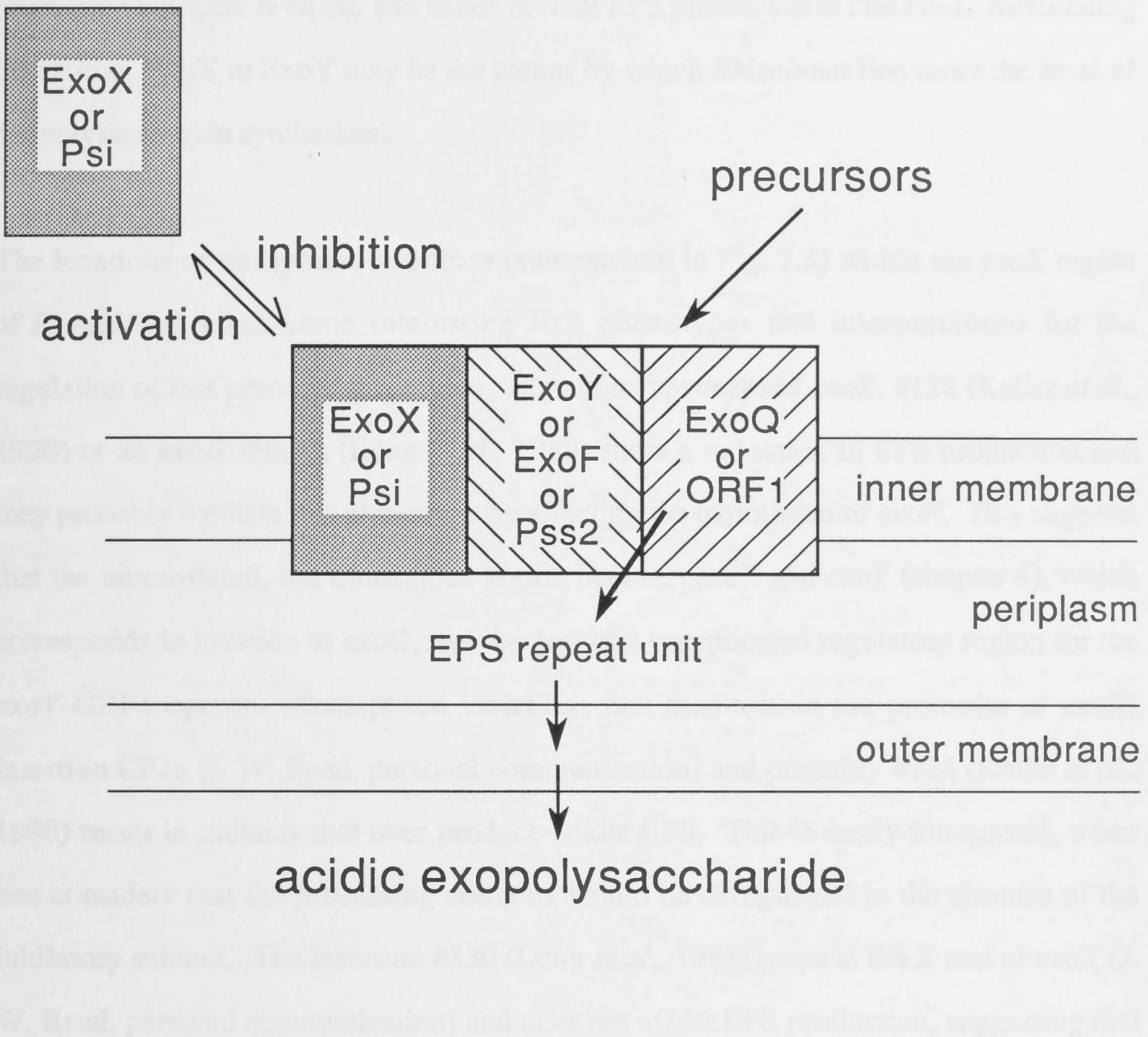

Fig. 7.2 Model of the proposed processing complex for EPS synthesis. The complex consists of at least two major components encoded by the *exoY*/*exoF*/*pss2* and ORF1/*exoQ* genes (depending upon the species). This membrane associated complex is the proposed site for the recognition and assembly of the oligosaccharide precursors. The regulatory component ExoX/Psi (depending upon the species) inhibits EPS synthesis only when it is bound to the complex. This proposed association is reversible.

the dosage of *exoY* is elevated along with *exoX*, the ratio of ExoX inhibitor to ExoY processing complex is equal, and hence normal EPS production is observed. Modulating the ratio of ExoX to ExoY may be the means by which *Rhizobium* fine tunes the level of exopolysaccharide synthesized.

The locations of transposon insertions (summarized in Fig. 7.3) within the *exoX* region of *R. meliloti* gives some interesting Exo phenotypes and interpretations for the regulation of this processing complex. Mutations upstream of *exoX*, #128 (Keller *et al.*, 1990) or an *exoG* mutant (Long *et al.*, 1988) show a reduction in EPS production and they probably mediate this phenotype by affecting the expression of *exoF*. This suggests that the untranslated, but transcribed region between *exoX* and *exoY* (chapter 4), which corresponds in location to *exoG*, may be part of a complicated regulatory region for the *exoY*-ORF1 operon. Transposon insertions that map within the promoter of *exoX*, insertion CF3a (J. W. Reed, personal communication) and probably #124 (Keller *et al.*, 1990) result in mutants that over produce acidic EPS. This is easily interpreted, when one considers that the processing complex would be deregulated in the absence of the inhibitory subunit. The insertion #330 (Long *et al.*, 1988) maps at the 3' end of *exoX* (J. W. Reed, personal communication) and does not affect EPS production, suggesting that the carboxy terminal of ExoX is not essential for the reversible binding characteristics of the polypeptide. However, the insertion site for the *exoJ* classification (Long *et al.*, 1988) maps between CF3a and #330 and this mutant is Exo^-. This observation suggests that an ExoX polypeptide, truncated to this point, is still capable of binding to the complex and thereby inhibiting EPS synthesis; however, it has lost the ability to disassociate. The irreversible binding of these truncated ExoX' polypeptides permanently inhibits EPS synthesis by these proposed complexes.

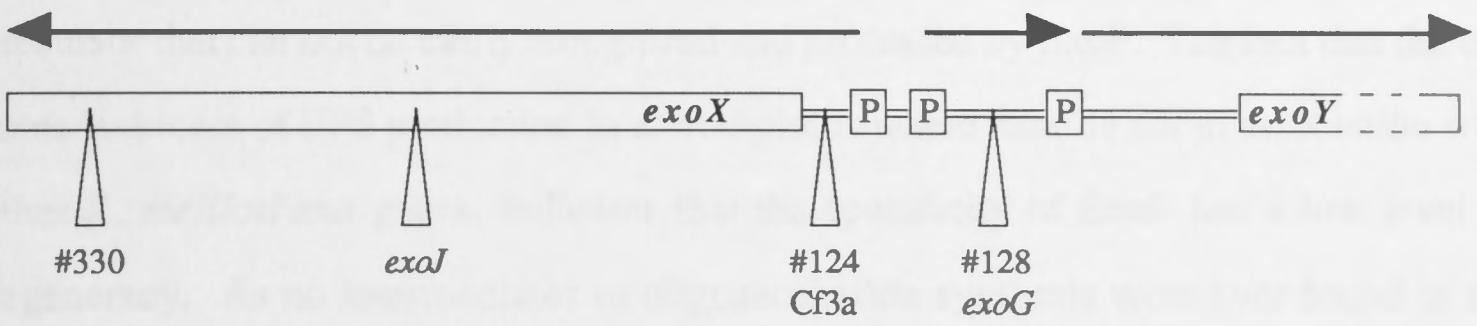

Fig. 7.3 Genetic map of Tn*5* insertion sites within the *R. meliloti exoX* region. Mutations #124 and #128 are from Keller *et al.* (1990) and mutations #330, CF3a, *exoJ* and *exoG* are from Long *et al.* (1988) and J. W. Reed (personal communication). Coding regions are represented by thick open bars and "P" denotes putative promoter regions.

Hybrid strains between *R.* sp. NGR234 (with a nonasaccharide repeat unit) and *R. meliloti* (with an octasaccharide repeat unit) has provided new insights into the functions of *exoY* and *exoF* (chapter 6). Merodiploids of *R.* sp. NGR234 *exoY* mutants carrying plasmids with the *R. meliloti exoF* gene in association with other *R. meliloti exo* genes (pRG100; *exoP,N,M,A,L,K,H,J,G,X,F,Q*), form EPS of the *R. meliloti* type (succinoglycan) and not the EPS of *R.* sp. NGR234. An *R. meliloti* plasmid (pD56) carrying *exoF* in the absence of other donor *exo* genes (*exoP,N,M,A,L,K,H*) was only able to confer a slight amount of EPS production to *exoY* mutants of *R.* sp. NGR234. Finally, merodiploid constructs of *R.* sp. NGR234 that have a wild-type *exoY* and are carrying a donor *exoF* in a association with other *R. meliloti exo* genes (pRG100) produce a mixture of the two exopolysaccharide structures with a predominance of the succinoglycan-type *R. meliloti* EPS. This suggests that the products of *exoY* (of strain NGR234) and *exoF* (of *R. meliloti*) are involved in specific

recognition of EPS precursors from their own species. For succinoglycan production from an ExoF complex, it is also necessary to provide other structural genes involved in its biosynthesis, which implies that the *exo* genes of *R.* sp. NGR234 synthesize an EPS precursor that can not be easily recognized and processed by ExoF. The fact that there is some leakiness of EPS production in merodiploids where *exoF* is not in association with other *R. meliloti exo* genes, indicates that the specificity of ExoF has a low level of degeneracy. As no intermediates to oligosaccharide synthesis were ever found in the analysis of *R.* sp. NGR234 *exoY* mutants (Djordjevic *et al.*, 1987b), it is concluded that the complex is involved in the earliest steps of transferring UDP-sugar residues to the lipid carrier in the membrane. Furthermore, as the assembly of the oligosaccharide follows an ordered process at the membrane, the completion of the repeat unit probably takes place at this complex as well.

The complicated function performed by ExoY or ExoF suggests that it is a subunit component of a larger complex, involving products from other genes. Downstream of *exoY*, in the same operon, is another gene termed ORF1 (chapter 4). Complementation analysis, indicates that the presence of *exoY* in the absence of ORF1 or another 3' cistron is deleterious (perhaps lethal) to the *Rhizobium* cell, although it was not necessary to maintain comparable gene dosage. ORF1 encodes a large protein and has extensive hydrophobic domains. In the *R. meliloti* complex, the equivalent role of ORF1 could be fulfilled by *exoQ*, as it is located downstream of *exoF* and has been implicated in the specific synthesis of succinoglycan (Long *et al.*, 1988). DNA sequence of this region by Keller *et al.* (1990) revealed a coding region named ORF-II (407 amino acids), which is probably *exoQ*. Therefore, the *exoQ*/ORFII equivalent in *R.* sp. NGR234 is probably ORF1, because each of these genes are immediately down stream and in the same operon as *exoF* or *exoY*, depending upon the strain. The proposed polypeptide sequence of ORF-II of *R. meliloti* has a region of homology to a sequence motif of 15 amino acids from the mouse testicular lactate dehydrogenase (Keller *et al.*, 1990). This motif is

responsible for binding the coenzyme NADH/H^+ (Pan *et al.*, 1980) and some biosynthetic reactions of EPS synthesis are known to be coupled with NADH hydrolysis reactions (Jarman and Pace, 1984).

The basis of this EPS processing-regulatory system is probably the same in all fast-growing *Rhizobium* species and other soil bacteria as well. The *exoX* gene from *R.* sp. NGR234 or *R. meliloti* can effectively inhibit EPS synthesis in *R. fredii, R. l.* bv. *phaseoli*, *R. l.* bv. *viciae* and *A. tumefaciens*, which all have DNA sequences homologous to *exoY* in their genomes. Furthermore, the fact that cloned DNA from *X. campestris* is able to correct the Exo^- and symbiotic phenotypes of *pss2* mutants of *R. l.* bv. *phaseoli* and *R. l.* bv. *viciae* (Borthakur *et al.*, 1986) is probably another example of degeneracy or leakiness associated with a foreign processing complex operating in a recipient cell.

There is potential for environmental conditions to influence EPS production at this processing complex. In *R. meliloti*, transcription of *exoF* is repressed by two other loci, *exoR* and *exoS*. Mutations in *exoR* and *exoS* result in deregulated over-production of EPS and evidence suggests that *exoR* is influenced by ammonia availability (Doherty *et al.*, 1988). Similarly, in *R.* sp. NGR234, transcription of *exoY* is repressed by the 2895 gene (chapter 5) and the colony morphology of strain ANU2895 is the same (Exo^{++}) as the *exoR*::Tn*5* and *exoS*::Tn*5* mutants of *R. meliloti*. However, regulation of the 2895 gene has not yet been investigated.

Another very likely environment sensing gene is *nodD2* from *R. fredii* (Appelbaum *et al.*, 1988). This gene does not regulate the *nodABC* operon in a manner generally associated with *nodD*, but instead *nodD2* inhibits EPS production in strains of *R. fredii* and *R.* sp. NGR234 when introduced into these strains on multicopy plasmids. As no sequence homology exists between *nodD2* and *exoX*, it is unlikely that *nodD2* acts as an

inhibitory subunit at the complex in a manner similar to that of *exoX*. However, analysis of the *exoY* promoter shows sequences reminiscent of *nod*-box sequences, which are the target sites for NodD proteins (Hong *et al.*, 1987; Fisher *et al.*, 1988; Kondorosi *et al.*, 1989b). It may be that in the presence of environmental stimuli or plant signals, the *nodD2* gene product may bind to this promoter and repress its transcription; with the net result being the inhibition of EPS synthesis in the same manner as that caused by the absence of *exoY*. The repression of *exoY* for the specific purpose of inhibiting EPS synthesis may occur during a later stage of nodule development, so that carbon can be allocated for nitrogenase activity rather than used in EPS production.

7.2 Activity of Exopolysaccharides During Infection

The specificity of action of polysaccharides indicates that they have more than a simple passive role in the establishment of symbiosis. For example, the second EPS (EPS-II or EPSb) of *R. meliloti* strain SU47 (Glazebrook and Walker, 1989; Zhan *et al.*, 1989) is sufficient for symbiosis with alfalfa, but not with four other plants that are normally hosts for *R. meliloti*. In addition, *exoH* mutants of *R. meliloti* (Leigh *et al.*, 1987) produce only large molecular weight polymers of the acidic EPS, which is not succinylated; these mutants are defective in nodule invasion and the growth of infection threads. Further evidence of the specificity of *Rhizobium* EPS was found in studies of transconjugants made with *R.* sp. NGR234 mutants and cosmids carrying *R. meliloti exo* genes (chapter 6). If *exoY* and the biosynthetic *exo* genes of strain NGR234 were deleted and replaced with *R. meliloti exo* genes (pRG100), then Exo^+ colonies were formed with EPS of the *R. meliloti* succinoglycan type, but these hybrid transconjugant strains were still defective in their nodulation of *Leucaena*. Thus, the production of the homologous EPS appears to be necessary for the successful Nod^+Fix^+ phenotypes induced by *R.* sp. NGR234. One possible explanation for this proposed specific biological activity of the oligosaccharides is that the different oligosaccharide ligands bind to specific membrane receptors at the root hair surface and are involved in the crucial step of initiating infection

threads. Different receptor molecules would be present in each plant and hence this would contribute to *Rhizobium* host-range. However, some plants which form determinate-type nodules are apparently unaffected by the structure of the *Rhizobium* acidic EPS. For example, beans, cowpeas, siratro and soybeans can be induced to form nitrogen fixing nodules by different strains of both *Rhizobium* and *Bradyrhizobium* bacteria, which have very different acidic EPS structures (Dudman, 1984).

7.3 Future Directions

The work and models presented in this thesis have opened up several avenues of logical progression for future experimentation regarding EPS biosynthesis in *R*. sp. NGR234. The cellular location of a putative membrane multi-meric complex for the biosynthesis of acidic EPS would be best examined by a biochemical approach. Transcriptional fusions of the coding regions for *exoX* and *exoY* to *E. coli* promoters would allow the production of large quantities of gene products and facilitate the generation of polyclonal antibodies to the proteins. Polyclonal antibodies may be more appropriate than monoclonal antibodies, since the presumptive interactions between ExoX, ExoY and possibly other proteins could cause different epitopes to be exposed under different circumstances. Such antibodies could be used to test for the *in situ* associations of ExoX and ExoY with each other and other unidentified subunits, by radio-immune detection assays of such complexes after electrophoresis through non-denaturing acrylamide gels and subsequent transfer to membrane filters. In addition, their prevalence in particular cell fractions could be examined in a similar way also using the specific antibodies. A biochemical study of protein interactions and the location of these proteins within the cell would either strengthen the model beyond little doubt or conversely necessitate a reworking of it.

The definite regulation of acidic EPS by the gene mutated with Tn*5* in strain ANU2895 and possible regulation by a *nodD2*-like gene similar to that found in *R. fredii*

(Appelbaum *et al.*, 1988) should also be investigated further. The wild-type allele for the 2895::Tn*5* locus could be cloned and then its mode of action for regulating EPS, possibly via *exoY*, could be characterized. The potential exists for the 2895 locus to be regulated by environmental conditions in a manner similar to that proposed for the nitrogen regulation of *exoR* in *R. meliloti* (Doherty *et al.*, 1988). The genome of *R.* sp. NGR234 should also be examined for the presence of *R. fredii nodD2*-like sequences, either using the *R.* sp. NGR234 *nodD* gene or the *R. fredii nodD2* gene as a probe, and then subsequently cloned if detected. The *nodD2* gene may be a plant environment sensing gene and/or a gene coordinating the expression of *nod* and *exo* genes. Its mode of action may be via the putative *nod*-box sequences in the promoter of *exoY* (chapter 6) and this could be tested by either precise deletion or site directed mutagenesis within the sequence, or by gel retardation assays.

Construction of hybrid *Rhizobium* strains that carry a cluster of foreign *exo* genes and are disabled only in the synthesis of their own acidic EPS, will be very useful tools for the study of acidic EPS involvement in host-range and the development of nitrogen-fixing nodules. Perhaps the first experiments should be a more thorough characterization of the 616-d deletion strains, with particular attention to the left-most extremity of the deletion. The new *Bam*HI fragment created by the deletion in strain 616-d could be cloned using the 10 kb *Bam*HI fragment of pJG11 as a probe. Using the newly cloned DNA, that was juxtaposed to the remaining *exoY* DNA at the right-end of the deletion, as a probe to test for homologous sequences in R'3222 or pRG100 will establish whether the limit of the deletion extends beyond the region of cloned DNA in both of these recombinant plasmids. If the deletion does extend beyond the cloned region of the complementing plasmids, then the possibility that other symbiotic genes or nitrogen fixation genes may have also been deleted, cannot be ruled out. A more precise deletion of DNA within the defined *exo* region would reduce the risk of deleting other non-*exo* symbiotic genes.

In the longer term, studies will investigate how the separate oligosaccharide-type signals from rhizobia are necessary for the successful infection of legumes and the establishment of a nitrogen-fixing nodule. Radio-labelling of oligosaccharides will be used to demonstrate the presence of specific membrane receptors in root hair cells and how the interaction leads to plant gene expression and the formation of a possible secondary messenger system, which results in root hair curling, infection thread formation and cortical cell division.

Publication

The model for biosynthesis of acidic EPS that was presented in this chapter, was also presented in the following review publication:-

Gray, J. X. and B. G. Rolfe. Exopolysaccharide production in *Rhizobium* and its role in invasion. *Molecular Microbiology*. **4**:1425–1431.

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